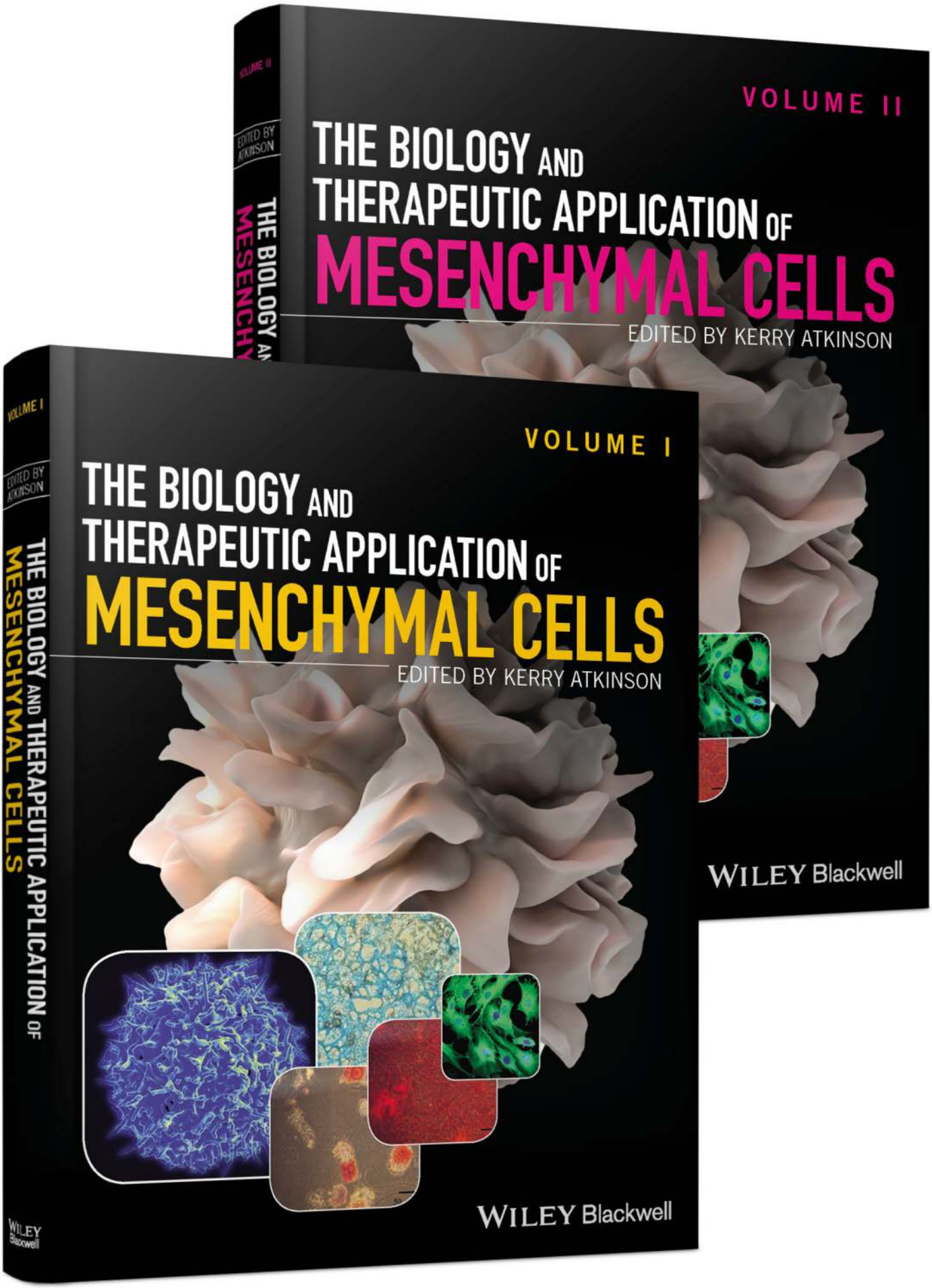

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- The BMT Data Book: A Manual for Bone Marrow and Blood Stem Cell Transplantation. K. Atkinson, Cambridge University Press, Cambridge, UK, 1998.
- Clinical Bone Marrow and Blood Stem Cell Transplantation. Second edition. Editor, K. Atkinson. Cambridge University Press, Cambridge, UK, 2000.
- Clinical Bone Marrow and Blood Stem Cell Transplantation. Third edition. Editors K. Atkinson, R Champlin, J Ritz, M Brenner, P Ljungman and W Fibbe. Cambridge University Press, Cambridge, UK, 2004.
- The BMT Data Book. Second edition. Editors, R Munker, H M Lazarus and K Atkinson, Cambridge University Press, Cambridge UK, 2009.
- The BMT Data Book: Including Cellular Therapy. Third edition. Editors, R Munker, C Hildebrandt, H M Lazarus and K Atkinson, Cambridge University Press, Cambridge, UK, 2013.
- Hematopoietic Cell Transplants. Concepts, Controversies and Future Directions. Editors H M Lazarus, R P Gale, A Keating, A Bacigalupo, R Munker, K Atkinson and A Syed. Cambridge University Press, Cambridge, UK, 2016. In press.
- Bird Facts for Everyone. Outskirts Press, Denver, USA, 2013.

# The biology and therapeutic application of mesenchymal cells

## Volume I and II

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**Kerry Atkinson**

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For Paris, Sarah, Jake, Jessica and Dylan

The wounded surgeon plies the steel

That questions the distempered part;

Beneath the bleeding hands we feel

The sharp compassion of the healer's art

Resolving the enigma of the fever chart.

T.S. Eliot. *Four Quartets*. East Coker. IV

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# Editor's preface

Serendipity plays a role in the lives of most, if not all, people. I was training in internal medicine at the Royal Sussex County Hospital in Brighton, UK, in 1970 when we had a number of young adults admitted with acute leukemia. We referred them to the Royal Marsden Hospital in Sutton, Surrey, which is a hospital that deals only with cancer. I got to know my opposite number there, Dr Sandy Patterson, and one day on the phone he asked me if I was interested in a job there. I said I was, had an interview, and got the job. During my rotation in the Adult Leukemia Unit run by Professor Gordon Hamilton-Fairley, Professor Tim McElwain, and Professor Ray Powles it quickly became evident to me that adults with acute leukemia, while they would often attain remission with combination chemotherapy, almost all relapsed and subsequently died. At the end of the 1960s and in the early 1970s Professor E Donnall Thomas and his team in Seattle, Washington, USA, began their pioneering work on allogeneic bone marrow transplantation for people with otherwise incurable hematologic malignancies. He received the Nobel Prize in Medicine for this work in 1990. Mac and Ray had the foresight to introduce this at the Royal Marsden, and so I got my first exposure to this therapy, which, although difficult and dangerous, miraculously cured some recipients, acting in part by the as then undescribed process known as the graft-versus-leukemia effect, mediated by T cells in the donor bone marrow. Amazing stem cell biology fact number 1: the donor's cells (usually) engraft and the reconstituted hematopoietic and immune systems in the recipient remain (usually) completely of donor origin. Thus, if a sister receives a hematopoietic stem cell (HSC) graft from her brother, her blood and immune system cells will carry an XY chromosome and not an XX chromosome. I still find this amazing. I was hooked and I did a 3 month fellowship at the Fred Hutchinson Cancer Research Center in Seattle before joining the staff working with Professor Rainer Storb in the laboratory and as an attending physician on the ward for 4 years. My wife Pauline is Australian and wanted to return there. Professor Jim Biggs had just started Australia's first bone marrow transplant program and I joined him at St Vincent's Hospital, Sydney, in 1981. In 1996 Dr Chris Juttner, who had been running an equivalent bone marrow transplant program in Adelaide, had become Vice-President of Clinical and Regulatory Affairs at Systemix Inc., a biotechnology company started by Professor Irv Weissman of Stanford University with its intellectual property wrapped around the $CD34^+$ $CD90^+$ phenotype of human HSCs. Chris asked me if I would consider running Systemix's HSC trials in the USA and Europe. This was an exciting if volatile experience. Systemix closed down 2 years later and I subsequently took a position as Vice President of Clinical and Regulatory Affairs at Osiris Therapeutics Inc., a biotechnology company in Baltimore, USA, whose intellectual property covered human mesenchymal stem cells (MSCs). Amazing stem cell biology fact number 2: I listened in astonishment as scientists at Osiris told me about the apparent lack of need for histocompatibility matching between MSC donors and MSC recipients, thus opening the door to the possibility of cryopreserved allogeneic MSC banks with an "off-the-shelf" approach to their use in clinical trials. This hooked me on MSCs and is the reason, 15 years later, for putting this book together.

It is interesting, and somewhat sobering, to compare the clinical translation of these two adult stem cell types, both discovered some 50 years ago.

After the discovery of the human leukocyte antigens (HLA) of the major histocompatibility complex (MHC), Dausset and van Rood recognized in 1958 that these antigens followed the Mendelian principles of inheritance. In 1968 Epstein and coworkers published their work on the development of antisera for typing dog leukocyte antigens (DLA). These reagents enabled Storb, Thomas, and their colleagues in Seattle to define the principles of bone marrow transplantation in large outbred animals. They used littermate beagle donor–recipient pairs that were either DLA-identical or nonidentical, as well as beagle donor–recipient pairs that were DLA nonidentical and unrelated to each other. They found that 10 Gy (1000 rads) of total body irradiation enabled donor marrow engraftment in littermate DLA-identical recipients. They examined an array of immunosuppressive agents to determine that methotrexate (at that time) was the optimal prophylaxis for graft-versus-host disease (GVHD) posttransplant. They found that antithymocyte globulin could be effective treatment for acute GVHD in some cases. With the principles established in the canine model, Thomas and the Seattle team began their clinical trials in allogeneic HSC transplantation at the end of the 1960s using sibling donor–recipient pairs who were genotypically identical for the HLA-A, -B, and -DR antigens. They found that allogeneic HLA-identical bone marrow transplantation could cure otherwise incurable hematologic malignancies. Gradually, a number of improvements resulted in allogeneic HSC transplantation becoming part of mainstream clinical medicine, where it remains. There was thus a relatively short interval between the discovery of HSCs and their translation to the clinic.

Apart from their biology, HSCs differ in two significant ways from MSCs. First, HSCs are relatively easy to procure; second, there is no intellectual property around them: they are in the public domain. In contrast, MSCs require simple but expensive *ex vivo* expansion to obtain enough numbers for clinical use – primarily of their progeny, mesenchymal stromal cells. Second, there is a swathe of intellectual property around them. These features have probably slowed their translation to the clinic, and at present, 50 years after their discovery by Friedenstein and colleagues, there is not yet a definitive role for them in mainstream clinical medicine. They have a fascinating biology, and research progress continues to expand exponentially.

There at least two potential alternatives to allogeneic MSC transplantation using unrelated MSCs from unrelated donors. One is the use of the autologous stromal vasculature fraction of fat tissue obtained by liposuction, concentrated by centrifugation and then administered directly to the patient who donated the fat. The cells present in the fraction include preadipocytes, mesenchymal stromal cells, endothelial progenitor cells, T cells, B cells, and mast cells. This approach received a boost in 2013 when the cell membrane markers of these cells were better defined (see Chapter 2). The fact that it is an autologous procedure and is minimally manipulated makes it a theoretically attractive candidate, at least when direct injection into a localized area is needed. However, like every other experimental therapeutic agent, they need to proceed through the normal channels of human research ethic committee scrutiny and, if approved, follow the phase I, II, and III clinical trial pathway.

At the other end of the scientific (and very recently clinical) spectrum is the MSC exosome (Chapters 19 and 38). These remarkable agents transport a cargo of mRNA, microRNAs, and proteins and deliver them to the appropriate target cell. Thus, acellular therapy, providing the MSCs' "goodies" without the need for the cells themselves may be possible. Will there develop a new medical specialty of exomology?

So much to do, so many avenues to explore. It will require scientists and clinicians working closely together. It will take time – witness the fact that HSC transplantation started in the clinic in the 1960s and 1970s and is still evolving. It will take the degree of erudition and hard work that the chapter authors in this book have displayed. I would like to acknowledge them for their scholarship and hard work and to thank all of them for their contribution. The book would be nothing without them. I would also like to thank Mindy Okura-Marszycki at John Wiley and Sons for championing the proposal to commission the book and Stephanie Dollan, Purvi Patel and Shikha Pahuja, Nuraiziah Johri who provided all the logistical support required to actually produce the book; in addition I would like to acknowledge the skill and hawk-like vision of Peter Lewis, the book's copy editor, who never failed to get back to me even while on holiday. I have learned an enormous amount from editing it.

Kerry Atkinson
Brisbane and Sydney
June 2016

SECTION I

# An overview of mesenchymal stem cells and mesenchymal stromal cells

CHAPTER 1

# The mesenchymal stem cell, the mesenchymal stromal cell, and the mesenchymal stromal cell exosome

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## 1.1 Nomenclature

This book is front-ended by a brief description of my own view of the "MSC" – stem cell or stromal cell or both – and is a polarized view. The book is back-ended by a much more nuanced and erudite interpretation written by Siddaraju Boregowda and Don Phinney of the two populations and allows for a neat amalgam of the two. This shows that the term "mesenchymal stem/stromal cell" is not as much of an exercise in hedge-sitting as I had previously viewed it. Drs Boregowda and Phinney make the excellent point that one cannot know what mesenchymal cells at what time in their development are present in a given bulk preparation unless detailed assays of true stem cells are carried out. This is often not practical and is never easy. However, I still find the term "mesenchymal stem/stromal cell" disconcerting when it is applied to studies using *ex vivo* expanded mesenchymal cells without any attempt being made to see if there are true mesenchymal stems cells contained within the population. If mesenchymal stromal cells are the subject of a chapter I have used mesenchymal stromal cells and their abbreviation "MSC." If mesenchymal stem cells are the subject of a chapter or part of a chapter I have referred to them as such and have not used any abbreviation for them.

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## 1.2 The mesenchymal stem cell

In 1970, Till and McCullagh in Canada discovered the hematopoietic stem cell (HSC) [1]. This was the first adult stem cell to be discovered. This discovery was quickly exploited clinically with the introduction in the late 1960s of allogeneic bone marrow transplantation using HLA-identical siblings as donors for people with hematologic malignancies refractory to conventional chemotherapy [2]. Other sources than bone marrow became available, such as peripheral blood stem cells mobilized out of the marrow and into the circulation by the administration of hematopoietic growth factors, several of which were discovered by Don Metcalf in Melbourne, Australia [3]. Subsequently, Eliane Gluckman in Paris demonstrated that blood taken from the umbilical cord immediately after the birth of the baby contained sufficient numbers of HSCs to allow this source to be used in HSC transplantation, at least in children [4]. HSC transplantation has been part of mainstream clinical practice for over 40 years. E. Donnall Thomas, who headed the Adult Leukemia Center at the Fred Hutchinson Cancer Research Center in Seattle, USA, where HSC transplantation was pioneered, was awarded the Nobel Prize in Medicine for this work in 1990.

Stem cells have two properties: they must be able to divide and be self-renewing and they must be able to differentiate down a specific lineage or set of lineages to produce functional cells. In the case of HSCs this was readily shown first in animal models and subsequently in humans that if the recipient's bone marrow was ablated by high-dose chemotherapy or chemoradiotherapy,

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donor bone marrow cells infused into the recipient after the high-dose treatment reconstituted the recipient's bone marrow both short term and long term. The HSCs infused were capable of both self-renewal and hematopoietic lineage differentiation to functional erythrocytes, white cells, and platelets. Thus, the recipient's hematopoietic and immune cells are of donor origin posttransplant. That meant that if you transplanted HSCs from a male donor into a female recipient and then karyotyped them, they would have an XY karyotype – and vice versa, a phenomenon that has amazed me ever since.

In the 1960s Alexander Friedenstein *et al.* in Russia discovered a second type of adult stem cell that he called colony-forming units fibroblastic (CFU-Fs), since single cells grew into colonies and were readily distinguishable from HSCs by their adherence to plastic and their fibroblast-like morphology [5]. Friedenstein and Owen called these cells osteoprogenitors. Subsequently, these cells were shown to differentiate to key elements of the skeleton, including bone (osteoblasts), cartilage (chondrocytes), fat (adipocytes), and periosteum (fibroblasts). Additionally, evidence of self-renewal has been shown by Sacchetti and colleagues for osteoprogenitor cells in bone marrow sinusoids that were able to organize a hematopoietic microenvironment [6]. They showed that MCAM/CD146-expressing subendothelial cells in human bone marrow stroma were capable of transferring, upon transplantation, the hematopoietic microenvironment to heterotopic sites, coincident with the establishment of identical subendothelial cells within a miniature bone organ. Establishment of subendothelial stromal cells in developing heterotopic bone marrow *in vivo* occurred via specific, dynamic interactions with developing sinusoids. Subendothelial stromal cells residing on the sinusoidal wall were found to be major producers of angiopoietin 1 – a pivotal molecule of the HSC "niche" involved in vascular remodeling. Their data revealed the functional relationships between establishment of the hematopoietic microenvironment *in vivo*, establishment of skeletal progenitors in bone marrow sinusoids and angiogenesis. Subsequently, Mendez-Ferrer *et al.* confirmed these findings by showing that mesenchymal and HSCs formed a unique bone marrow niche [7]. This area of science was well summarized by Paolo Bianco and colleagues in 2008 [8], 2011 [9], and 2013 [10]; and see Chapter 32.

In 2013, Chan *et al.* took this field several stages further when they described a lineage-restricted, self-renewing common skeletal progenitor (bone, cartilage, and stromal progenitor cell) isolated from the limb bones and bone marrow tissue of fetal, neonatal, and adult mice [11]. This cell clonally produced chondrocytes (cartilage-forming) and osteogenic (bone-forming) cells and at least three subsets of stromal cells that exhibited differential expression of cell surface markers, including CD105 (endoglin), CD90 (Thy-1) and 6C3 (ENPEP glutamyl aminopeptidase (aminopeptidase A)). These three stromal subsets exhibited differential capacities to support hematopoietic (blood-forming) stem and progenitor cells. Although the 6C3-expressing subset demonstrated functional stem cell niche activity by maintaining primitive HSC renewal *in vitro*, the other stromal populations promoted HSC differentiation to more committed lines of hematopoiesis, such as the B cell lineage. Gene expression analysis and microscopic studies revealed a microenvironment in which $CD105^{-}$, Thy-$1^{-}$, and 6C3-expressing marrow stromal cells collaborated to provide cytokine signaling to HSCs and more committed hematopoietic progenitors. As a result, within the context of bone as a blood-forming organ, this bone/cartilage/stromal progenitor cell was found to play a critical role in supporting hematopoiesis through its generation of diverse osteogenic and hematopoietic-promoting stroma, including HSC supportive $6C3^{+}$ niche cells.

Thus, the term *skeletal stem cell* for these cells is well founded scientifically and, therefore, bone or bone marrow mesenchymal cells should be the best candidate cells for repair of disorders of the skeletal system.

True mesenchymal stem cells are rare cells with a frequency in bone marrow of between 1 in $10^4$ and 1 in $10^5$. To enable both experimental and clinical trials of mesenchymal cells to be performed, it has been necessary to expand them in *in vitro* cultures. The cells obtained are not mesenchymal stem cells, but rather their progeny – often mistakenly called mesenchymal stem cells but in fact they are mesenchymal stromal cells or mesenchymal progenitor cells and their tissue of origin should be indicated, for example bone marrow mesenchymal stromal cells. They are also known as CFU-F as indicated above in Friedenstein's initial descriptions of them, in which case their organ or tissue of origin should be included – for example, bone marrow CFU-F, cardiac CFU-F, adipose tissue CFU-F, and so on.

Table 1.1 compares and contrasts allogeneic hematopoietic stem cells with allogeneic mesenchymal stem cells.

In the rest of this chapter the term mesenchymal stromal cells will be used and the abbreviation "MSCs" will be applied only to this term.

## 1.3 The mesenchymal stromal cell

While it has been shown that MSCs can differentiate *in vivo* to bone and tendon, they have also been shown to be immunomodulatory of both the innate and adaptive immune systems (see Chapters 33 and 34). Additionally, MSCs have been shown to mediate therapeutic benefit in a wide range of animal models of disease and in clinical trials in many diseases apart from musculo-skeletal disorders. It has been noted in many studies that detection of administered MSCs is difficult beyond a few days after their administration, and there is evidence that they can mediate a therapeutic benefit by secretion of proteins appropriate for repair of tissue or organ damage. Thus, they have been described by Caplan and colleagues as an injury drug store [12,13]. Additionally, they do not circulate in blood but have been found to be perivascular cells surrounding the endothelial cells [14]. Thus, they can be obtained from any vascularized tissue. Presumably this is how they "work" in damaged

**Table 1.1** Comparison of allogeneic hematopoietic stem cells with allogeneic mesenchymal stem cells.

| Parameter | Allogeneic hematopoietic stem cell | Allogeneic mesenchymal stem cell |
|---|---|---|
| Year discovered | 1961 | 1966 |
| Years to first clinical trial | 9 | 32 |
| Safe | No – can cause fatal graft-versus-host disease | Safe |
| Effective | Yes – can cure otherwise incurable diseases | Unknown. Phase III clinical trial results awaited. Likely to be useful in disorders of the musculo-skeletal system |
| Part of mainstream clinical medicine | Yes | No |
| Easy to harvest | Yes – from bone marrow, mobilized peripheral blood, or cord blood banks | No – Require careful selection (see Chapter 32) |
| Enmeshed in intellectual property rights | No | Yes |

organs or tissues – they are already present at the site of injury as long as that tissue is vascularized. While it is thought that all MSCs are pericytes, not all pericytes are MSCs; for example, smooth muscle cells form part of the vessel wall of arterioles, arteries, and larger veins. Despite their presence in almost all tissues it is of interest that they have a tropism for inflamed tissue and will home to such tissue if injected intravenously (see Chapters 22–24).

In 2006 the International Society for Cell Therapy proposed the following minimal criteria for the definition of MSCs. First, MSCs must be plastic-adherent when maintained in standard culture conditions (Figures 1.1 and 1.3). Second, MSCs must express CD105 (endoglin), CD73 (lymphocyte-vascular adhesion protein 2 (L-VAP-2), also known as 5′ nucleotidase) and CD90 (Thy-1), and must lack expression of CD45 (a pan-leukocyte marker), CD34 (a sialomucin present on hematopoietic stem cells and endothelial cells), CD14 (a co-receptor for the detection of bacterial lipopolysaccharide), CD11b (integrin alpha M, ITGAM), CD79A (which identifies the B cell antigen receptor complex-associated protein alpha chain and MB-1 membrane glycoprotein), CD19 (also a B cell antigen), and the major histocompatibility complex class II molecules. Third, MSCs

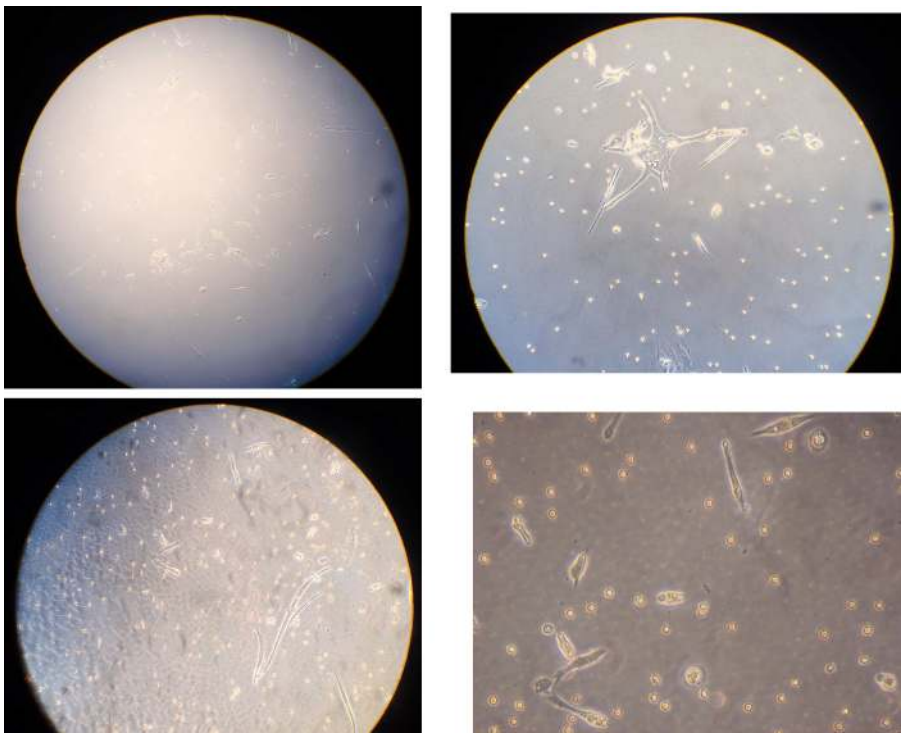


**Figure 1.1** Human mesenchymal stromal cells derived from term placenta in culture at passage 1.

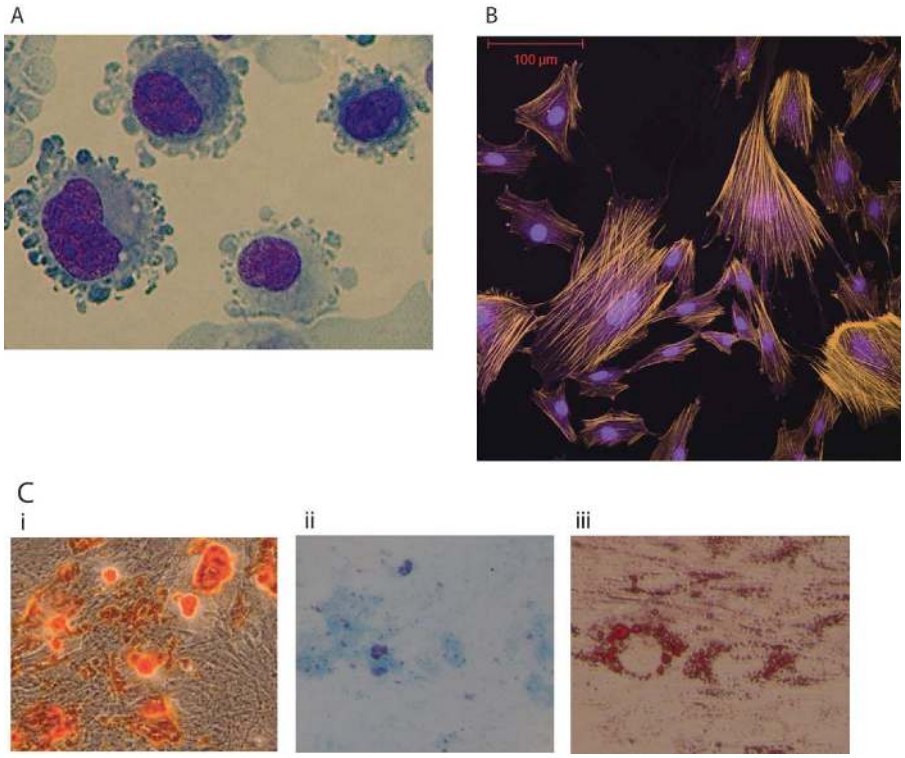



**Figure 1.2** (A) Giemsa staining of a suspension of human MSCs showing densely packed eccentric nuclei and vacuolation of the cytoplasm. The blebs around the edges of the cells are thought to be artifactual and induced by the cytospin preparation. Magnification ×400. (B) The actin cytoskeleton of human MSCs. (C,i). Adipogenic differentiation of human MSCs induced *in vitro* and stained with Oil Red O; (C,ii). Chondrogenic differentiation of human MSCs induced *in vitro* and stained with alcian blue. (C,iii). Osteogenic differentiation of human MSCs induced *in vitro* and stained with alizarin red. Photograph courtesy of Dr Tony Rossetti. (See color plate section.)

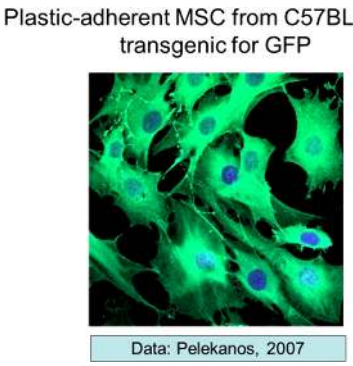



**Figure 1.3** Murine bone/bone-marrow-derived MSCs from mice transgenic for green fluorescence protein illustrating their fibroblastoid appearance and adherence to plastic. Nuclei stained blue with DAPI. Photograph courtesy of Dr Rebecca Pelekanos. (See color plate section.)

must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro* [15] (Figure 1.2 ci, cii and ciii).

This area took another significant step forward with the publication in 2013 of a joint statement from the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy on stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells (ADSCs) [16].

The centrifuged but uncultured stromal vascular fraction was identified as being $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$ (platelet endothelial cell adhesion molecule), and $CD34^+$, while cell surface positivity for CD13 (alanine aminopeptidase), CD73, CD90, and CD105 were best used in conjunction with CD45, CD235a, and CD31 [16].

*Ex vivo*-expanded ADSCs retain cell surface markers expressed by other mesenchymal stromal/stem cells, including CD44 (the hyaluronic acid receptor), CD73, CD90, and CD105, and are also negative for CD45 and CD31, but can be distinguished from bone-marrow-derived MSCs by being $CD36^+$ (fatty acid translocase, also known as glycoprotein IIIb) and $CD106^-$ (vascular cell-adhesion molecule 1) [16].

## 1.4 The mesenchymal stromal cell exosome and extracellular vesicles

Extracellular vesicles (EVs) are membrane vesicles that are released by many cell types into the extracellular space and consist of several classes, including exosomes, microvesicles, ectosomes, membrane particles, and exosome-like vesicles or apoptotic bodies (see Chapters 20 and 38 for detailed descriptions). However, the defining parameters for each of these different classes are not definitive, and the use of the terms exosomes, microvesicles, and microparticles is often ambiguous and not rigorously qualified. Generally, EVs can be formed by either inward budding of endolysosomal vesicles followed by exocytosis (e.g., exosomes), or shedding from the plasma membrane (e.g., microvesicles). They have attracted much interest

from the biomedical research community because of their potential as sources of biomarkers for diseases, therapeutic agents, or vehicles for drug delivery. Generally, EVs carry a cargo of proteins and nucleic acids that reflect their cell of origin and the pathologic or physiologic state of their cell source. In addition, these EVs with their cargoes could be internalized by other cells as a form of intercellular communication, making these vesicles potential therapeutic agents or delivery vehicles of therapeutic agents.

The exosome is the best characterized of all the EVs described to date and can be differentiated from other EVs by its biogenesis. The exosome is the only EV known to be derived from endosome, and this endosomal origin of exosomes is reflected in its membrane, which is enriched for lipids commonly found in lipid rafts such as cholesterol, sphingomyelin, and ceramide. Lipid rafts are specialized microdomains in the plasma membrane where much of the cellular endocytosis takes place to form endosomes. Subsequent invaginations of the endosomal membrane generate numerous intraluminal vesicles (ILVs) within the multivesicular body (MVB). If the MVB fuses with the plasma membrane, the ILVs are released as exosomes. The process by which cellular proteins and RNAs are selected and loaded into exosomes has not been elucidated. MSC exosomes have been among the best characterized exosomes to date (Chapters 20 and 38).

Recently, MSC exosomes were administered to a person with treatment-refractory grade IV (severe) acute graft-versus-host disease who went into remission with a dramatic alleviation of her disease symptoms and who remained stable for 5 months [17]. If confirmed in clinical trials this might represent a way to exploit the benefits of MSCs without having to administer the cells themselves.

## References


1 E.A. McCulloch and J.E. Till. The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. *Radiat Res* 1961;**13**:115–125.

2 E.D. Thomas, C.D. Buckner, M. Banaji, *et al*. One hundred patients with acute leukemia treated by chemotherapy, total body irradiation, and allogeneic marrow transplantation. *Blood* 1977;**49**:511–533.

3 D. Metcalf. The colony stimulating factor. *Aust J Exp Biol Med Sci* 1972;**50**:547–557.

4 E. Gluckman, A. Devergie, H. Bourdeau-Esperou, *et al*. Transplantation of umbilical cord blood in Fanconi's anemia. *Nouv Rev Fr Hematol* 1990;**32**:423–425.

5 A.J. Friedenstein, I.I. Piatetzky, and K.V. Petrakova. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;**16**: 381–390.

6 B. Sacchetti, A. Funari, S. Michienzi, *et al*. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–336.

7 S. Mendez-Ferrer, T.V. Michurina, F. Ferraro, *et al*. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

8 P. Bianco, P.G. Robey, and P.J. Simmons. Mesenchymal stem cells: revisiting history, concepts and assays. *Cell Stem Cell* 2008;**2**: 313–319.

9 P. Bianco. Back to the future: moving beyond "mesenchymal stem cells". *J Cell Biochem* 2011;**112**:1713–1721.

10 P. Bianco, X. Cu, P.S. Frenette, *et al*. The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;**19**:35–42.

11 C.K. Chan, P. Lindau, W. Jiang, *et al*. Clonal precursor of bone, cartilage, and the haematopoietic niche. *Proc Nat Acad Sci U S A* 2013;**110**:12643–12649.

12 A.I. Caplan and D. Correa. The MSC: an injury drugstore. *Cell Stem Cell* 2011;**9**:11–15.

13 N.G. Singer and A.I. Caplan. Mesenchymal stem cells: mechanisms of inflammation. *Ann Rev Pathol Mech Dis* 2011;**6**:457–478.

14 L. da Silva Meirelles, A.I. Caplan, and N.B. Nardi. In search of the *in vivo* identity of mesenchymal stromal cells. *Stem Cells* 2008;**26**:2287–2299.

15 M. Dominici, K. Le Blanc, I. Mueller, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

16 P. Bourin, B.A. Bunnell, L. Casteilla, *et al*. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics (IFATS) and Science and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013;**15**:641–648.

17 L. Kordelas, V. Rebmann, A.K. Ludwig, *et al*. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014;**28** (4):970–973.

CHAPTER 2

# The nomenclature of mesenchymal stem cells and mesenchymal stromal cells

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## 2.1 Introduction

Nomenclature is important in science, not least because it sometimes channels research based on the assumptions that underlie the names we ascribe to cells or biological processes. It can be imagined that if terminology is inaccurate or misleading, advances could be delayed or misdirected. The nomenclature of mesenchymal stem cells and mesenchymal stromal cells has evolved with the field and has been influenced considerably at various times by the prevailing scientific controversies surrounding these cells.

## 2.2 Historical perspective

Friedenstein's seminal studies in the 1960s and 1970s laid the groundwork for the mesenchymal stem cell and mesenchymal stromal cell field. It was not unreasonable that early in the studies he termed the cells mechanocytes or osteogenic stem cells and then marrow stromal cells, but in subsequent work they became known as marrow fibroblasts, underscored by the terminology of his assay of the mesenchymal stromal cell progenitor, CFU-F (colony forming unit–fibroblast) [1].

Subsequent work in the 1970s by Michael Dexter and others identified an adherent cell population present in cultured bone marrow cells that could maintain hematopoiesis for many weeks [2]. Ongoing hematopoiesis *in vitro* was demonstrated with human long-term bone marrow cultures that also depended on an adherent marrow stromal cell population [3]. During the 1980s the term *marrow stromal cells* (abbreviated to MSCs) became well established and was used to distinguish the population from adherent hematopoietic cells such as macrophages and from marrow fibroblasts, which were considered phenotypically and functionally distinct from mesenchymal stem cells and mesenchymal stromal cells by the inability of the former to support long-term hematopoiesis *in vitro* [4].

At that time, research with marrow stromal cells focused on understanding the interactions taking place in this *in vitro* model of the hematopoietic microenvironment and in the processes mediating hematopoiesis. The area was challenging, and advances with the model were relatively limited. During this period, scientific productivity plateaued.

It was in this context that the proposal in 1991 by Arnold Caplan to consider marrow stromal cells as *mesenchymal stem cells* (also then abbreviated to MSCs) captured the attention of many investigators in the field [5], especially after the publication by Pittenger and colleagues in 1999 on the "multilineage potential of adult human mesenchymal stem cells" [6].

This coincided with other studies suggesting the ability of hematopoietic and other cells to undergo transdifferentiation. Although numerous reports supported the notion of the ability of mesenchymal stem or stromal cells to undergo differentiation to cells not only of mesodermal origin, but also of endodermal and even ectodermal origin, almost all lacked evidence of the acquisition of the functional characteristics of the differentiated cells [7]. Moreover, other work refuted earlier reports showing that hematopoietic cells undergo transdifferentiation to cardiac and other cell types [8].

Despite these formidable caveats and the lack of convincing mechanistic data, a consequence of the use of the term "stem" by

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many investigators in the field was the promotion of the notion that mesenchymal stem or mesenchymal stromal cells could be used as cell *replacement* therapy. An explosion in research on the potential role of these cells in tissue regeneration ensued and continues to the present. However, it has been shown definitively by Méndez-Ferrer *et al.* [9] and Sachetti *et al.* [10] that non-hematopoietic cells in the bone marrow of mice could form new bone on serial transplantation into recipient mice and that this new bone was populated by the recipient's hematopoietic cells. These studies showed that there was indeed a population of rare mesenchymal cells in murine marrow that fulfilled the criteria of stem cells by being able to produce daughter stem cells and to differentiate along at least one line of mesodermal differentiation – osteogenesis. This gave rise to yet another name: skeletal stem cells.

## 2.3 The need for common terminology and definition: the International Society for Cellular Therapy white papers of the mid-2000s

A major concern among investigators in the field in the early 2000s was the lack of a common definition of mesenchymal stem cells or mesenchymal stromal cells among the many papers published in the field. This dilemma was further reflected by the absence of an accepted terminology for the cell population and the persistent notion that the cells under study were invariably *stem cells*. It became increasingly difficult, if not impossible, to compare the results of studies. In response, the Non-hematopoietic Mesenchymal Cell Committee of the International Society for Cellular Therapy (ISCT) prepared two white papers, published in *Cytotherapy*, to address these limitations. The first paper was a clarification of the terminology, emphasizing that while most mesenchymal stem or stromal cells are not stem cells, the bulk population represents a multipotent mesenchymal stromal cell (MSC) population [11]. The second white paper provided minimal criteria for the definition of MSCs [12]. These were as follows. First, MSCs must be plastic-adherent when maintained in standard culture conditions. Second, MSCs must express CD105 (endoglin), CD73 (lymphocyte-vascular adhesion protein 2 (L-VAP-2), also known as 5′ nucleotidase) and CD90 (Thy-1), and must lack expression of CD45 (a pan-leukocyte marker), CD34 (a sialomucin present on hematopoietic stem cells and endothelial cells), CD14 (a co-receptor for the detection of bacterial lipopolysaccharide), CD11b (integrin alpha M), CD79A (which identifies the B cell antigen receptor complex-associated protein alpha chain and MB-1 membrane glycoprotein), CD19 (also a B cell antigen), and the major histocompatibility complex class II molecules. Third, MSCs must differentiate to osteoblasts, adipocytes, and chondroblasts *in vitro*.

This area took another significant step forward with the publication in 2013 of a joint statement from the International Federation for Adipose Therapeutics and Science and the ISCT on stromal cells from the adipose-tissue-derived stromal vascular fraction and culture expanded adipose-tissue-derived stromal/stem cells (ADSCs) [13].

The centrifuged but uncultured stromal vascular fraction was identified as being $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$ (platelet endothelial cell adhesion molecule) and $CD34^+$, while cell surface positivity for CD13 (alanine aminopeptidase), CD73, CD90, and CD105 were best used in conjunction with CD45, CD235a, and CD31 [13].

*Ex-vivo*-expanded ADSCs retain cell surface markers expressed by other mesenchymal stromal/stem cells, including CD44 (the hyaluronic acid receptor), CD73, CD90, and CD105, and are also negative for CD45 and CD31, but can be distinguished from bone-marrow-derived MSCs by being $CD36^+$ (fatty acid translocase, also known as glycoprotein IIIb) and $CD106^-$ (vascular cell-adhesion molecule 1) [13].

## 2.4 Updating terminology

It has become increasingly apparent that both the nomenclature and definition of mesenchymal stromal cells requires updating. The field has continued to evolve, and over the past decade more evidence has accumulated that MSCs exert effects on tissue regeneration by mechanisms independent of the regeneration of new tissue. Because of the plethora of anti-inflammatory, anti-apoptotic, pro-angiogenic, and immunosuppressive proteins secreted by MSCs, Dr Caplan later suggested the term "medicinal signaling cell" to describe them – also abbreviated to "MSC." The abbreviation "MSC" in this book, however, refers only to mesenchymal stromal cells.

MSCs can now be derived from many different tissues and exhibit differences in immunophenotype and functional characteristics. To help chart the update, a consortium of international MSC investigators held a series of workshops to address the challenges facing the field, including a reassessment of MSC nomenclature. The consensus of the Nomenclature Working Group has recommended the following terminology for mesenchymal stromal cells [13]: donor type (autologous or allogeneic), species of origin (e.g., human, mouse), tissue source (e.g., bone marrow, adipose tissue, cord blood), mesenchymal cell type (stem cell, stromal cell, or progenitor cell). An example of this would be human allogeneic cord-blood-derived mesenchymal stromal cells.

In summary, it appears that there is indeed a rare population, at least in bone marrow, that fulfills the criteria of mesenchymal stem cells. In addition, there are the undifferentiated progeny of these cells that can be expanded massively in *in vitro* culture and are termed mesenchymal stromal cells or mesenchymal progenitor cells or multipotent mesenchymal stromal cells.

This exciting field is likely to benefit further from adoption of a common nomenclature that more precisely defines the cells under study.

## References


1 Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**:393–403.

2 Dexter, T.M., Allen, T.D., and Lajtha, L.G. Conditions controlling the proliferation of haemopoietic stem cells in vitro. *J Cell Physiol* 1977;**91**,335–344.

3 Gartner, S. and Kaplan, H.S. Long-term culture of human bone marrow cells. *Proc Nat Acad Sci U S A* 1980;**77**:4756–4759.

4 Keating, A., Powell, J., Takahashi, M., and Singer, J.W. The generation of human long term marrow cultures from marrow depleted of Ia (HLA-DR) positive cells. *Blood* 1984;**64**:1159–1162.

5 Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* 1991;**9**:641–650.

6 Pittenger, M.F., Mackay, A.M., Beck, S.C., *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**: 143–147.

7 Keating, A. Mesenchymal stromal cells: new directions – a perspective. *Cell Stem Cell* 2012;**10**:709–716.

8 Balsam, L.B., Wagers, A.J., Christensen, J.L., Kofidis, T., Weissman, I.L., and Robbins, R.C. Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. *Nature* 2004;**428**: 668–673.

9 Méndez-Ferrer, S., Michurina, T.V., Ferrao, F., *et al*. Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

10 Sacchetti, B., Funari, A., Michienzi, S., *et al*. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–336.

11 Horwitz, E., Le Blanc, K., Dominici, M., *et al*. Clarification of the nomenclature for MSC: the International Society for Cellular Therapy position statement. *Cytotherapy* 2005;**7**:393–395.

12 Dominici, M., Le Blanc, K., Mueller, I., *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**:315–317.

13 Bourin, P., Bunnell, B.A., Casteilla, L., *et al*. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics (IFATS) and Science and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013;**15**:641–648.

SECTION II

# The isolation and *ex vivo* expansion of mesenchymal stromal cells

CHAPTER 3

# The isolation and expansion of mesenchymal stromal cells from bone marrow

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## 3.1 Introduction

Cell-based therapies (other than hematopoietic stem cell (HSC) transplantation, which has been part of mainstream medicine for over four decades) are a new wave in the development of biological agents to treat or cure a wide range of medical conditions, including hereditary, acquired, and degenerative human diseases [1]. Stem cells or their differentiated progeny appear to be powerful candidates for cell therapy and cell-based gene therapy since they have the capacity to undergo extensive replication in an undifferentiated state as well as to proliferate along committed lines of differentiation. They have the potential to differentiate either *in vitro* or *in vivo* into distinct tissues, making them an attractive candidate for clinical applications especially in the field of regenerative medicine.

Mesenchymal stem/stromal cells (MSCs) are a heterogeneous population of multipotent cells that can differentiate into the mesenchymal lineages of fat, bone, tendon, muscle, and cartilage [2]. They have the ability to modulate oxidative stress and secrete cytokines and growth factors that have immunomodulatory, anti-inflammatory, angiogenic, and anti-apoptotic effects [2]. Increasingly, evidence is focusing on these paracrine effects rather than transdifferentiation as a mechanism for MSC-mediated effects in both preclinical models and clinical applications.

MSCs are also of interest because they preferentially migrate to sites of inflammation and injury when injected systemically (see Chapters 22 and 23). They can also be transplanted across major histocompatibility complex (MHC) barriers into immune competent recipients without the need for immune suppression of the recipient before or after MSC administration. This enables them to be cryopreserved as an allogeneic off-the-shelf cellular therapy available for immediate use in acute or chronic medical settings. These remarkable qualities have encouraged clinical trials of MSCs in a range of differing medical conditions with some promising early results. Indeed, two MSC products have received marketing approval by national regulatory authorities: one for the repair of chronic non-union bone fractures using autologous bone-marrow-derived MSCs (in Australia) and one for treatment-refractory acute graft-versus-host disease following allogeneic HSC transplantation in children using allogeneic bone-marrow-derived MSCs (in Canada).

MSCs have been traditionally derived from bone marrow. However, as humans and animals increase in age there is a decrease in MSC production in the bone marrow. Additionally, bone marrow aspiration is a painful procedure. Crisan *et al.* reported that MSCs were a component of the endothelium and thus are present in all vascularized organs [3]. Other tissue sources, such as adipose tissue derived from liposuction aspirates [4–6] and gestational products, including the placenta [7,8] and umbilical cord [9] obtained at term delivery of the baby, have been reported as alternative sources [10]. These alternative sources are more easily accessible than bone marrow, generally less invasive, and larger amounts of MSCs can be obtained compared with bone marrow. Although MSCs from different tissue sources share many biological characteristics, functional

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and gene expression differences between MSCs from different sources have been reported [11–13]. Some of these differences, such as cell surface phenotype, differentiation potential, transcriptome/proteome characteristics, and immunomodulatory activity, represent specific features of MSCs from a specific tissue source [14–17], while others reflect the heterogeneity of MSC populations from different organs [18]. Still other differences may simply be due to different isolation and culture protocols.

## 3.2 Stem cells

Stem cells are characterized by two major attributes: their unlimited capacity to self-renew and their potential to give rise to progeny that can differentiate into specialized cell types. A true stem cell must be able to self-renew while maintaining an identical and undifferentiated state. In addition, they must be able to differentiate into multiple cell types. Their degree of possession of these qualities determines how potent these cells are.

Stem cells exist at different hierarchical levels in the development of an organism (Figure 3.1). There are pluripotent or totipotent stem cells that occur at one end of the spectrum. These cells arise during fertilization and have the ability to form an entire organism (e.g., the fertilized oocyte, embryonic stem cells, or induced pluripotent stem cells). This type of stem cell can replace cells or tissues from any organ or tissue. However, owing to their unlimited self-renewal and differentiation potential, tumor (teratoma) formation can occur, which increases the risk of using progeny derived from them for clinical application if not all of the original stem cells have differentiated. Examples of such stem cells are embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). Deriving differentiated tissues and organs from ESCs destroys the embryo, and this has generated significant ethical debate. iPSCs are generated in the laboratory and are not part of the mammalian body. They are not, however, associated with the ethical concerns surrounding ESCs.

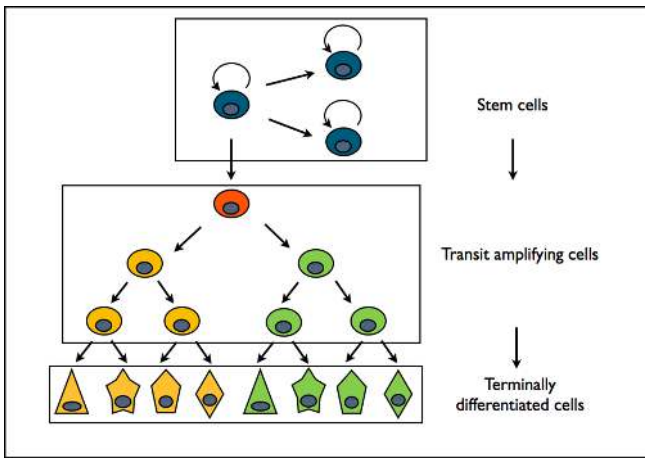



**Figure 3.1** The different hierarchical levels of stem cell potency. Image adapted from Perez-Losada and Balmain [19].

At the other end of the spectrum are adult stem cells, better considered as postnatal stem cells, whose differentiation capability is restricted to the formation of cell types within a single tissue lineage. These cells are multipotent, not pluripotent or totipotent, stem cells and do not form teratomas. Thus, these cells are at present safer for clinical use, albeit more limited in their potential application. HSCs are examples of such stem cells. They can self-renew, as demonstrated by their ability to reconstitute the hematopoietic and immune systems of animals, including humans, whose marrow has been ablated by high-dose total body irradiation and chemotherapy. They can, however, only differentiate to blood and immune system cells [20].

Mesenchymal stem cells are another type of adult multipotent stem cells and were discovered by Friedenstein and his colleagues in the 1960s. The term mesenchymal stem cell was coined by Caplan in 1991 as these cells were able to differentiate into multiple cell types of the mesenchymal lineage (including bone, cartilage, and fat) [21]. However, at this time there was a lack of convincing evidence that such cells could self-replicate into daughter stem cells. This changed when Sacchetti and colleagues demonstrated in 2007 the existence of self-renewing, plastic-adherent $CD146^+$ bone marrow stem cells using serial transplantation experiments in immunocompromised mice [22]. These mesenchymal stem cells are rare, with a frequency in bone marrow of 1 in $10^4$ cells to 1 in $10^5$ cells. In order to secure sufficient numbers of MSCs to perform experiments or for clinical transplantation it is necessary to expand them *ex vivo*, and the cells that are produced are not stem cells as defined previously here [23]. True self-renewing stem cells constitute only a fraction, if any, of such populations, with the majority of cells so produced being a heterogeneous population of progenitor cells called mesenchymal stromal cells, mesenchymal progenitor cells, or transit amplifying cells [24].

In 2005 the International Society for Cellular Therapy proposed that bone-marrow-derived plastic-adherent cells, generally described as 'mesenchymal stem cells,' should be renamed "mesenchymal stromal cells" [23]. Thus, the remainder of this chapter will discuss mesenchymal stromal cells and the abbreviation MSC will be used to denote this type of cell.

## 3.3 Isolation and characterization of bone marrow mesenchymal stromal cells

MSCs were first discovered in the bone marrow by Friedenstein more than 40 years ago [25]. These cells give rise to tissues of mesodermal lineage, including bone, cartilage, muscle, tendons, and adipose tissue [26–33]. Bone-marrow-derived MSCs are a rare cell population (~0.001%) of bone marrow mononuclear cells *in vivo*, resulting in a low yield upon initial isolation. Therefore, as already indicated, *ex vivo* expansion of these cells

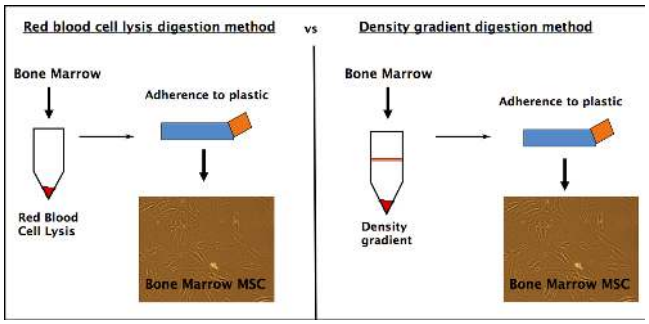



**Figure 3.2** Comparison of the red blood cell lysis and density gradient digestion methods for the isolation of bone-marrow-derived MSCs.

is required to gain sufficient numbers for laboratory experiments or clinical trials. The isolation method consists of the whole marrow being subjected to fractionation using a density gradient such as Percoll for animals or Ficoll in humans. Alternatively, red blood cell lysis can be performed and the cells are then cultured in basal medium such as Dulbecco's modified Eagle's medium and 10–20% fetal calf–bovine serum [18,34–36]. Subsequently, cells are maintained in culture for several days during which contaminating nonadherent hematopoietic cells such as macrophages are depleted (Figure 3.2).

In some instances the property of plastic adherence alone is not sufficient to allow purification of MSCs, as in the case of cells derived from murine bone/bone marrow. Phinney *et al.* [37] reported the presence of hematopoietic $CD45^+$ $CD11b^+$ granulocytic and monocytic precursors in these cultures. Owing to the lack of specific phenotypic markers, these observations led to the development of new methods involving CD34, CD45, and CD11b immunodepletion to generate purified bone marrow MSC preparations [38].

### 3.3.1 Cell surface markers

The International Society for Cell Therapy developed the following criteria for identifying human MSCs [39]. First, MSCs must be plastic-adherent when maintained in standard culture conditions using tissue culture flasks. Second, 95% or more of the MSC population must express the cell surface markers CD105, CD73, and CD90, which are characteristic but not unique markers for MSCs. Additionally, these cells must lack cell surface expression (<2% positive) of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA class II. Third, the cells must be able to differentiate to osteoblasts, adipocytes, and chondrocytes under *in vitro* differentiating conditions. Other cell surface antigens also characteristic of, but not unique to, MSC populations obtained from various sources include nestin [40], CD29, CD44, CD49b, CD130, CD146, CD166, CD271, CD200, and αV/β5 integrin [3,41–43].

Simmons and other workers suggested that expression of Stro-1 and VCAM-1 can be used to preferentially enrich for multipotent MSCs [44–46]. In addition, it has been reported that CD271 on bone marrow MSCs selects for a subpopulation that has higher clonogenicity, increased paracrine secretion of cytokines, and enhanced engraftment properties [42,47]. However, the phenotype of MSCs can change during *ex vivo* expansion, and this may represent alterations in the biological features of the MSC population involved (see Chapter 7). For example, Stro-1 is present on freshly isolated bone marrow MSCs as well as other tissues. However, expression of Stro-1 is lost from MSCs during *ex vivo* expansion and it cannot be considered a unique MSC marker [48]. Indeed, even at the clonal level there remains diversity within these populations.

Murine MSCs derived from crushing the long bones and obtaining a cellular population from a combination of bone and bone marrow also lack specific cell surface markers, thus requiring multiple cell surface marker co-expression to assist in identification. Murine MSCs have been reported to express Sca-1, CD90, CD29, CD44, CD49e, CD51, CD81, CD24, CD105 and lack expression of CD45, CD11b, CD31, and CD117 [49]. They also display a transient cell surface phenotype related to the duration of *ex vivo* expansion in culture. The loss of CD90, CD15, and CD309 has been associated with *ex vivo* expansion [50]. The lack of a unique marker or markers for MSCs is a limitation in current MSC research.

We compared the cell surface markers present on adult human bone marrow-derived MSCs with those on term placenta-derived MSCs and with those on umbilical cord-blood-derived unrestricted somatic stem cells (USSCs) (Table 3.1). Based on a standard panel of MSC-descriptive antibodies, all the lines were virtually identical. All expressed CD29, CD44, CD49d, CD49e, CD73, CD90, CD105, CD146, CD49e, and CD166. All three cell populations were negative for Stro-1, CD45, CD34, CD50, CD106, and the pluripotency markers SSEA-4, Tra 1–60, and Tra1-81 [51].

Despite lacking a specific cell surface marker or markers, MSCs are unique among nucleated mammalian cells in that they stimulate little or no allogeneic reactivity when administered to MHC-unmatched adult immune competent recipients, perhaps due to their lack, at least in the human, of expression of co-stimulatory cell surface molecules such as CD40, CD80, or CD86 that are involved in the activation of T cells that mediate rejection of foreign tissues [52–56]. Furthermore, they are actively suppressive of T cell, NK cell, dendritic cell, and B cell function [52,53,57].

### 3.3.2 Chemokine receptor display

We also compared the cell surface and intracellular chemokine receptor display on adult human bone-marrow-derived MSCs with those on term-placenta-derived MSCs and with those on cord-blood-derived USSCs (Table 3.2). The surface and intracellular chemokine displays of bone marrow MSCs and placental MSCs were very similar. USSCs differed from bone marrow MSCs and placental MSCs by showing positive staining for the presence of intracellular CCR8, CCR10, and CCR11 [51].

**Table 3.1** Cell surface markers on adult human bone-marrow-derived MSCs (BM-MSCs), human term placenta-derived MSCs, and USSCs. Reproduced from Matigian *et al.*, *Genomics Data* 2015; **3**: 70–74 [51] under a Creative Commons License (CC BY). doi:10.1016/j.gdata.2014.11.011.

| Marker association | CD number/ name | Other name | BM-MSCs | Placental MSCs | USSCs |
|---|---|---|---|---|---|
| Mesenchymal stromal cell-associated markers | CD29 | Integrin-β1 | Positive | Positive | Positive |
| | CD44 | HCAM (homing cell adhesion molecule) | Positive | Positive | Positive |
| | CD73 | Ecto-5′-nucleotidase | Positive | Positive | Positive |
| | CD90 | Thy-1 | Positive | Positive | Positive |
| | CD105 | Endoglin | Positive | Positive | Positive |
| | CD146 | MCAM (melanoma cell adhesion molecule) | Positive | Not done | Positive |
| | GD2 | Neural ganglioside 2 | Faint staining | Faint staining | Faint staining |
| Mesenchymal stem-cell-associated marker | Stro-1 | Stromal cell molecule-1 | Negative | Negative | Negative |
| Endothelial marker | CD31 | PECAM-1 (platelet/endothelial cell adhesion molecule) | Negative | Negative | Negative |
| Hematopoietic cell-associated markers | CD34 | Mucosialin | Negative | Negative | Negative |
| | CD45 | Leukocyte common marker | Negative | Negative | Negative |
| Other adhesion molecules | CD49d | VLA-4 (very late antigen-4) | Positive | Positive | Positive |
| | CD49e | VLA-5a (very late antigen-5a) | Positive | Positive | Positive |
| | CD50 | ICAM-3 (intercellular adhesion molecule) | Negative | Faint staining | Negative |
| | CD166 | ALCAM (activated leucocyte cell adhesion molecule | Positive | Positive | Positive |
| Pluripotency markers | SSEA-4 | Stage-specific embryonic antigen-4 | Negative | Negative | Negative |
| | TRA 1–60 | | Negative | Negative | Negative |
| | TRA 1–81 | | Negative | Negative | Negative |

**Table 3.2** Chemokine receptor display by bone marrow MSCs, placental MSCs, and UCCSs. Reproduced from Matigian *et al.*, *Genomics Data* 2015; **3**: 70–74 [51] under a Creative Commons License (CC BY). doi:10.1016/j.gdata.2014.11.011.

| Chemokine receptor | BM-MSC surface display | BM-MSC intracellular display | Placental MSC cell surface display | Placental MSC intracellular display | USSC cell surface display | USSC intracellular display |
|---|---|---|---|---|---|---|
| CCR1 | Faint staining | Positive | Faint staining | Positive | Negative | Faint staining |
| CCR3 | Negative | Positive | Negative | Positive | Negative | Positive |
| CCR5 | Negative | Negative | Negative | Negative | Negative | Negative |
| CCR8 | Faint staining | Negative | Faint staining | Negative | Negative | Positive |
| CCR10 | Negative | Negative | Negative | Negative | Negative | Positive |
| CCR11 | Faint staining | Negative | Faint staining | Negative | Negative | Positive |
| CXCR3 | Negative | Positive | Negative | Positive | Negative | Positive |
| CXCR4 | Positive | Positive | Positive | Positive | Negative | Positive |

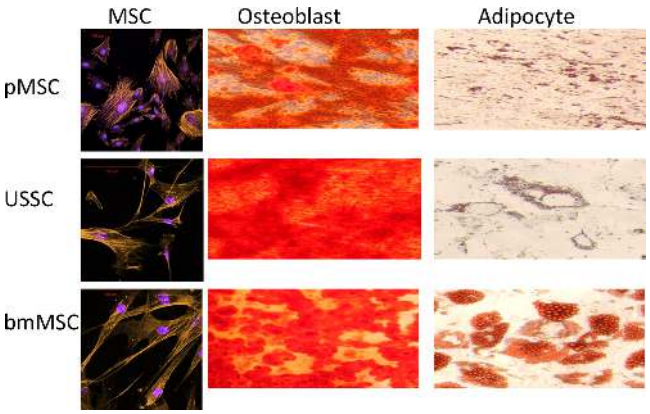



**Figure 3.3** Comparison of mesodermal differentiation to osteogenesis and adipogenesis by adult human bone marrow (bm)-derived MSCs, human term placenta (p)-derived MSCs, and USSCs from umbilical cord blood. Reproduced from [51], Matigian *et al.*, *Genomics Data* 2015; **3**: 70–74 under a Creative Commons License (CC BY). doi:10.1016/j.gdata.2014.11.011. (See color plate section.)

### 3.3.3 Mesodermal differentiation capability

We also compared the *in vitro* mesodermal differentiation potential of adult human bone-marrow-derived MSCs with those on term-placenta-derived MSCs and with those on cord-blood-derived USSCs (Figure 3.3). All three populations showed marked ability to differentiate to the osteogenic lineage. Bone marrow MSCs showed stronger differentiation to the adipogenic pathway than placental MSCs and USSCs, each of which showed only a slight degree of adipogenesis [51].

## 3.4 The immunomodulatory properties of mesenchymal stromal cells

The immunomodulatory properties of adult bone marrow MSCs have been extensively studied [58]. Secretion of soluble factors – including IL-1β [59], TGF-β1 [60], hepatocyte growth factor [60], prostaglandin $E_2$ [61], indoleamine 2,3-dioxygenase [62], heme oxygenase-1 [63], leukemia inhibitory factor [64], insulin-like growth factor (IGF) [65], soluble human leukocyte antigen G5 [66], galectin [45], and Jagged-1 [67] – have all been suggested as responsible for mediating the immunomodulatory effects of MSCs.

Several studies have demonstrated that both undifferentiated and differentiated bone marrow MSCs have suppressive effects on alloantigen and mitogen-stimulated lymphocyte proliferation in vitro, with a concomitant reduction in the production of proinflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α [54,68].

To date the interactions between adult bone marrow MSCs with T lymphocytes have been the most extensively studied. Many reports have demonstrated that adult bone marrow MSCs affect several properties of T cells, including suppression of the proliferation of activated $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells [69,70]. In addition, bone marrow MSCs can prolong the survival of unstimulated T cells by rescuing the lymphocytes from activation-induced cell death through down-regulation of Fas receptor and the Fas ligand on T cells as well as by inhibition of endogenous proteases involved in cell death [71]. Other studies have shown that bone marrow MSCs reduce IFN-γ production by $CD4^+$ Th1 cells and interleukin (IL)-17 release by $CD4^+$ Th17 lymphocytes, while IL-4 secretion by $CD4^+$ Th2 cells is increased [72,73].

Crisan *et al.* reported that MSCs migrate in response to digested extracellular matrix and other chemotactic stimuli, which recruit MSCs from both local surrounding environments to the site of injury or inflammation [3]. It is likely that increased inflammatory chemokine concentration at the site of inflammation is a major factor causing MSCs to migrate to these sites. Chemokines are released after tissue damage, and MSCs are known to express the receptors for many of these [74,75], including the chemokine receptor CXCR4, which is important in MSC migration [76–78]; and see Chapters 22 and 23. Furthermore, Ceradini *et al.* showed that the attraction of MSCs expressing CXCR4 to injured tissue is facilitated by hypoxia-inducible factor-1 (HIF-1), which causes overexpression of the CXCR4 ligand, stromal cell-derived factor-1 (SDF-1, also known as CXCL12) in a gradient proportional to tissue hypoxia [79]. A decline in HIF-1 expression has been associated with increasing age and may be responsible, at least in part, for the impaired ability of MSC migration to wound healing in the elderly [40].

As indicated earlier, it has been reported that both humans and animals demonstrate a decreased rate of production of bone marrow mesenchymal stem and progenitor cells with increasing age [80,81]. Several studies have provided evidence of a strong correlation between donor age and the proliferative potential exhibited by MSCs *in vitro* [81–84]. Thus, the MSC progenitor pool may be depleted following extensive proliferation. These findings correlate with *in vivo* studies of aging individuals [85,86]. A dramatic decrease in MSCs in bone marrow is observed from infants to the elderly, and a direct functional correlation exists between advanced age and decreased osteogenic potential [86,87]. This may contribute to diseases in the aging population, such as osteoporosis [84]. In addition, isolated MSCs with lower proliferative potential from older donors may be responsible for the reduced wound healing potential that is also associated with the aging population [88]. Such a decline in the quality of the cells is suboptimal for therapeutic applications.

For such reasons, alternative sources of MSCs are being investigated for clinical use in diseases in which the regenerative and/or immunomodulatory functions of MSCs may be useful [35,62,89,90]. A readily available and younger source that can be obtained by a noninvasive procedure, and which yields large numbers of MSCs for *ex vivo* expansion, would be an ideal alternative to adult bone marrow. One such tissue is term placenta (see Chapters 4 and 5).

Until recently the exact anatomical location of MSCs had not been well defined *in vivo*. It has now been established that MSCs

can be isolated from all vascularized tissues in the body since MSCs reside in the walls of blood vessels, forming part of the endothelium [3,41,91,92]. While MSCs are one form of pericyte, not all pericytes are MSCs: smooth muscle cells also form part of the endothelial wall in arterioles, arteries, and large veins. Several studies have indicated that MSCs also reside in specific niches in the bone marrow, where they provide essential support for HSCs – see Chapter 32. Thus, MSCs contribute to tissue, hematopoiesis, and immune system homeostasis [41,92].

Although bone marrow is the traditional source of MSCs, other sources are easily accessible, less invasive, and contain large numbers of MSCs [7,8,93–95]. MSCs have been isolated from first-trimester fetal liver, blood, and bone marrow [86], and broadly comparable cells have been isolated from a range of fetal and adnexal gestational tissues. MSCs have been isolated from amniotic fluid [96,97], umbilical cord blood [98], umbilical cord, including Wharton's jelly and perivascular tissue [99,100], first-trimester [101] and second-trimester liver, lung, bone marrow [102], and pancreas [103], term placenta [7], and amniotic and chorionic placental membranes [104]. Gestational sources can be collected at delivery or from prenatal diagnostic specimens such as amniotic fluid. The latter can be sourced by the minimally invasive technique of amniocentesis or obtained peripartum from tissue that would usually be discarded [105].

Though the minimal criteria for classifying MSCs have been established and many of these MSC populations share biological characteristics, functional differences between some of them have been reported [11–13]. These differences include cell surface phenotype, differentiation potential, and immunomodulatory activity and represent specific features of MSCs from different tissue sources [14–17], while others are reflective of differences in cell isolation and culture protocols [18]. These functional discrepancies between MSC populations have led to further characterization of these cells by a global, genomics-based approach whereby functional differences between MSC populations are reflected in their transcriptome. These findings have highlighted the need for a reproducible classification of a common gene expression profile for MSC populations regardless of their tissue source, and one that is distinct from that of differentiated cell types.

## 3.5 The transcriptome of mesenchymal stromal cells

Studies have reported the identification of the core MSC genomic profile and their functional pathways. A study by Shi *et al.* compared the gene expression profiles of human dental pulp and that of human bone marrow MSCs [106]. They reported that these MSC populations had a similar level of gene expression for approximately 4400 known human genes. Although they did not report all these genes, they mentioned genes that encoded extracellular matrix proteins (collagen types III and V, osteopontin, osteonectin, matrix-gla protein, decorin, biglycan, and alkaline phosphatase), cellular adhesion molecules (VCAM-1, CD44, integrins $\beta_1$, $\alpha_{v2}$, $\alpha_v$, and $\beta_3$), growth factors (IGF-1, PDGF, FGF-2, TGF-β1, BMP-2, BMP-4, and BMP-7), and transcription regulators (CBFA1,SX1, Msx2, c-FOS, c-JUN, and JUN-B).

These molecules are associated with the development and function of osteoblasts and/or odontoblasts and are indicative of cellular differentiation reflective of their tissue environment. Menicanin *et al.* isolated and characterized clonal populations of bone marrow, dental pulp, and periodontal-ligament-derived MSC populations to identify potential biomarkers associated with the long-term survival of multipotent MSCs [107]. They identified 24 genes, including the transcription factors E2F2, PTTG1, TWIST-1 and transcriptional cofactor LDB2 that were expressed in common between the MSC populations. These molecules could be grouped into categories based on their involvement in the cell cycle, mitosis, cell division, DNA repair and replication, and cellular differentiation.

A study by Wagner *et al.* compared MSCs from umbilical cord blood, adipose tissue, and bone marrow [17]. The primary aim of their study was to analyze the reproducibility of generating different MSCs under standardized conditions and to compare the gene expression profiles of MSCs derived from different sources. They found 25 upregulated genes that were common to all three MSC populations, and these included the genes for fibronectin, ECM2, glypican-4, ID1, NF1B, HOXA5, and HOXB6. Many of these genes are involved in extracellular matrix, morphogenesis, and development.

Al-Nbaheen *et al.* [11] performed a comparative study of adult adipose tissue, skin, and bone-marrow-derived MSC populations. They reported 6533 genes commonly expressed in each of these populations. Using a microarray-based approach they reported a common MSC molecular signature composed of 33 CD markers, including known MSC markers and several novel markers such as CD165, CD276, and CD82.

CD165 is a 37–42 kDa transmembrane glycoprotein, also known as AD2, gp37, and SN2. It is expressed on immature thymocytes, most platelets, thymic epithelial cells, fibroblasts, and acute T lymphoblastic leukemia cells. CD165 has also been found to be expressed on monocytes and circulating T cells as well as on islet cells of the pancreas, Bowman's capsule of the kidney, and on central nervous system neurons. The biological function of CD165 is still not clear, though it has been proposed that CD165 plays a role in cell adhesion between thymocytes and thymic epithelial cells during development.

CD276 (B7-H3) is a type I transmembrane protein belonging to the B7 family of co-stimulatory proteins. CD276 is mostly expressed on professional antigen-presenting cells, including dendritic cells, macrophages, and B cells. Initial studies showed that CD276 provides a stimulatory signal to T cells. However, more recent studies suggest a negative regulatory role for CD276 in T cell responses. Mouse CD276 protein inhibited T cell activation and effector cytokine production. CD276 is involved in the suppression of Th1-mediated immune responses and

played an important role in the development of pathogenic Th2 cells in a murine asthma model. Monoclonal antibody against B7-H3 enhances T cell proliferation *in vitro* and leads to exacerbated experimental autoimmune encephalitis *in vivo*.

CD82 is a 45–90 kDa type III tetraspan membrane protein. A member of the 4-span transmembrane protein superfamily (TM4SF), CD82 forms a complex with CD37, CD53, CD81, ECM, and MHC molecules. CD82 is expressed on monocytes, granulocytes, lymphocytes, epithelial cells, endothelial cells, and fibroblasts and plays a role in signal transduction and adhesion.

Significant differences in the molecular phenotype between the three different MSC populations in this study were observed, suggesting ontological and functional differences between the three populations. Al-Nbaheen *et al.* concluded that MSC populations obtained from different tissues exhibit significant differences in their molecular phenotype and suggested that this should be taken into consideration when planning their use in clinical protocols.

Tsai and colleagues also performed a comparative functional analysis from the gene expression signatures of fetal-derived MSCs (amniotic-membrane-derived MSCs, amniotic fluid MSCs, and umbilical cord blood MSCs) and adult MSCs derived from bone marrow [108]. They categorized the core genes found in common into five main groups related to plasmin-related extracellular matrix remodeling, cytoskeletal regulation, chemokine regulation and adhesion, plasmin activation, and TGF-β receptor signaling. Plasmin is an enzyme present in blood that degrades many blood plasma proteins, including fibrin clots.

Weng *et al.* also compared the transcriptomes of fetal umbilical cord blood and Wharton's jelly MSCs with that of adult bone-marrow-derived MSCs [109]. Their findings were similar to those of Tsai *et al.* and Menicanin *et al.*, in that cell adhesion molecules and Wnt signaling pathway genes were found to be significantly enriched in all the MSC populations. When they compared the gene set with gene ontology classification, the top four themed biological processes for adult bone-marrow-derived MSCs were immune system processes, biological adhesion, developmental processes, and locomotion. Fetal MSC processes included metabolic processes, cellular processes, developmental process, and signaling. Though there are differences in the molecules reported, these functional processes are consistent with previous studies of extracellular matrix remodeling, cellular and tissue development, and differentiation. In addition, a major network in fetal MSCs consisted of 547 genes that were involved in cell cycle development, RNA posttranscriptional modification, including RNA splicing, and DNA replication, recombination, and repair. They also reported that the targets of several microRNAs, including miR-124, miR-1, miR-199a1, miR-17, and miR-181b2, were enriched in fetal MSCs.

Miranda and colleagues observed that highly expressed genes in both human bone-marrow-derived MSCs and human umbilical cord vein MSCs can be modulated by microRNAs, which in turn can change protein abundance [110]. Their results showed that MSCs from both sources were similar in metabolic and functional processes relevant to their therapeutic potential, especially in immune system processes, their response to stimuli, migration and adhesion.

Similarly, Spitzer *et al.* studied the transcriptome and signaling pathways of endometrial MSCs [111]. They used fibroblasts and endothelial cells for comparison. They reported that endometrial MSCs expressed genes involved in angiogenesis/vasculogenesis, steroid hormone/hypoxia responses, inflammation, immunomodulation, cell communication, proteolysis/inhibition, and inhibition of Notch, TGF-β, IGF, Hedgehog, and G-protein-coupled receptor signaling pathways, which are involved in self-renewal and lineage specification pathways.

Studies have also compared MSC populations derived from alternative species to humans. A study by Monaco *et al.* compared porcine adipose-tissue-derived MSCs with bone-marrow-derived MSCs [112]. They reported a Pearson correlation coefficient between the complete transcriptome of adipose and bone marrow MSCs of 0.79 ($p < 0.0001$). When these MSC populations were compared with differentiated tissues, they reported that the significantly enriched functions of MSCs were related to protein synthesis with roles in extracellular matrix formation, differentiation, immune suppression, and cell cycle regulation.

Our group compared the gene expression profiles of multipotent human stromal cells isolated from cord blood, human term placenta and adult human bone marrow and showed distinct differences in gene expression patterns [51].

Pelekanos *et al.* conducted a comparative study of murine kidney, heart, and bone marrow MSCs. Some 81.1% of genes were expressed across the combined set of samples with the Pearson correlations of pairwise comparisons ranging from 0.878 to 0.924, thus indicating a high level of similarity between all MSC populations [18]. The majority of the top 100 genes encoded were housekeeping proteins. However, also included were regulatory molecules and secreted proteins involved in tissue-remodeling, repair, and inflammatory modulation, with the most highly expressed genes being zyxin, thymosin β10 and macrophage inhibitory factor. Zyxin may function as a messenger in the signal transduction pathway that mediates adhesion-stimulated changes in gene expression and may modulate the cytoskeletal organization of actin bundles. Thymosin β10 has direct inhibitive effects on endothelial migration and tube formation.

Other commonly expressed genes included biglycan, Serpinh1/HSP47, SerpinF1, also known as pigment epithelium-derived factor (PEDF), Sparc/Osteonectin, and CTGF/CCN2. Biglycan is a small leucine-rich repeat proteoglycan that is found in a variety of extracellular matrix tissues, including bone, cartilage, and tendon. Biglycan interacts with collagen both via the core protein and GAG chains. It is believed to play a role in the mineralization of bone. Knockout mice that have had the gene for biglycan suppressed have an osteoporosis-like phenotype with reduced growth rate and lower bone mass than mice that can express biglycan. Serpins are a group of

proteins that were first identified as a set of proteins able to inhibit proteases. The acronym serpin was originally coined because many serpins inhibit chymotrypsin-like serine proteases (serine protease inhibitors). The first members of the serpin superfamily to be extensively studied were the human plasma proteins antithrombin and antitrypsin, which play key roles in controlling blood coagulation and inflammation. Heat shock protein 47 (HSP47) is a member of the serpin family and is a collagen-specific molecular chaperone. Knockdown of HSP47 in murine models of fibrosis decreases the accumulation of collagen in fibrotic tissues and prevents the promotion of fibrosis. On the other hand, mutations in HSP47 cause collagen-related genetic diseases such as osteogenesis imperfecta. SerpinF1, also known as PEDF, is a multifunctional secreted protein that has antiangiogenic, antitumorigenic, and neurotrophic function. Osteonectin, also known as secreted protein acidic and rich in cysteine (SPARC), is a glycoprotein in the bone that binds calcium. It is secreted by osteoblasts during bone formation, initiating mineralization and promoting mineral crystal formation. Osteonectin also shows affinity for collagen in addition to bone mineral calcium. CTGF, also known as CCN2 or connective tissue growth factor, is a matricellular protein of the CCN family of extracellular matrix-associated heparin-binding and intercellular signaling proteins. CTGF has important roles in many biological processes, including cell adhesion, migration, proliferation, angiogenesis, skeletal development, and tissue wound repair and is critically involved in fibrotic disease. The acronym CCN is derived from the first three members of the family discovered, namely CYR61 (cysteine-rich angiogenic protein 61 or CCN1), CTGF (connective tissue growth factor or CCN2), and NOV (nephroblastoma overexpressed or CCN3). Together with three Wnt-induced secreted proteins, they comprise the CCN family of matricellular proteins. These proteins have now been renamed CCN1-6 by international consensus.

Functional pathways commonly expressed between these populations included immunity, cell adhesion, BMP signaling, chemokine signaling, homeodomain transcription factors, and metalloproteinases and their TIMP inhibitors.

Though these studies reported many similarities in gene expression and function pathways, there were discrepancies in the genes generated between the studies. Such discrepancies may be the outcome of variables such as tissue sources, isolation and culture expansion conditions, as well as array platforms and methods of analysis. To eliminate bias, a database of global gene expression profiles from published MSCs regardless of tissue source, culture conditions, array platform, or species needs to be established to then compare the commonly expressed genes within different MSC populations. The identification of genes that are commonly upregulated in all preparations of MSCs may serve as a novel parameter for the definition and characterization of MSCs. The identification of a core MSC transcriptome will be critical for improving our understanding of the mechanisms involved in the properties of these cells and is likely to influence parameters such as choice of MSC tissue source for clinical use (See Breaking News, Chapter 63). However, it is reassuring that, to date, no serious safety issues have been noted in the clinical use of MSCs [113].

## References


1 Brooke, G., Cook, M., Blair, C., *et al.* Therapeutic applications of mesenchymal stromal cells. *Semin Cell Dev Biol* 2007;**18**(6):846–858.
2 Deans, R.J. and Moseley, A.B. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000;**28**(8):875–884.
3 Crisan, M., Yap, S., Casteilla, L., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.
4 Priya, N., Sarcar, S., Majumdar, A.S., and SundarRaj, S. Explant culture: a simple, reproducible, efficient and economic technique for isolation of mesenchymal stromal cells from human adipose tissue and lipoaspirate. *J Tissue Eng Regen Med 2014*: **8**(9):706–716.
5 Zuk, P.A., Zhu, M., Ashjian, P., *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**(12):4279–4295.
6 Zuk, P.A. Stem cell research has only just begun. *Science* 2001;**293** (5528):211–212.
7 Yen, B.L., Huang, H.I., Chien, C.C., *et al.* Isolation of multipotent cells from human term placenta. *Stem Cells* 2005;**23**(1):3–9.
8 In't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., *et al.* Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 2004;**22**(7):1338–1345.
9 Kogler, G., Sensken, S., Airey, J.A., *et al.* A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004;**200**(2):123–135.
10 Da Silva Meirelles, L., Chagastelles, P.C., and Nardi, N.B. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**(Pt 11):2204–2213.
11 Al-Nbaheen, M., Vishnubalaji, R., Ali, D., *et al.* Human stromal (mesenchymal) stem cells from bone marrow, adipose tissue and skin exhibit differences in molecular phenotype and differentiation potential. *Stem Cell Rev* 2012,**9**:32–43.
12 Wislet-Gendebien, S., Laudet, E., Neirinckx, V., *et al.* Mesenchymal stem cells and neural crest stem cells from adult bone marrow: characterization of their surprising similarities and differences. *Cell Mol Life Sci* 2012;**69**(15):2593–2608.
13 Dmitrieva, R.I., Minullina, I.R., Bilibina, A.A., *et al.* Bone marrow- and subcutaneous adipose tissue-derived mesenchymal stem cells: differences and similarities. *Cell Cycle* 2012;**11**(2):377–383.
14 Izadpanah, R., Trygg, C., Patel, B., *et al.* Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J Cell Biochem* 2006;**99**(5):1285–1297.
15 Noel, D., Caton, D., Roche, S., *et al.* Cell specific differences between human adipose-derived and mesenchymal-stromal cells despite similar differentiation potentials. *Exp Cell Res* 2008;**314**(7):1575–1584.
16 Park, H.W., Shin, J.S., and Kim, C.W. Proteome of mesenchymal stem cells. *Proteomics* 2007;**7**(16):2881–2894.
17 Wagner, W., Wein, F., Seckinger, A., *et al.* Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 2005; **33**(11):1402–1416.
18 Pelekanos, R.A., Li, J., Gongora, M., *et al.* Comprehensive transcriptome and immunophenotype analysis of renal and cardiac

MSC-like populations supports strong congruence with bone marrow MSC despite maintenance of distinct identities. *Stem Cell Res* 2012;**8**(1):58–73.

19 Perez-Losada, J. and Balmain, A. Stem-cell hierarchy in skin cancer. *Nat Rev Cancer* 2003;**3**:434–443.

20 Yang, G., Hisha, H., Cui, Y., *et al.* A new assay method for late CFU-S formation and long-term reconstituting activity using a small number of pluripotent hemopoietic stem cells. *Stem Cells* 2002;**20**(3): 241–248.

21 Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* 1991;**9**(5): 641–650.

22 Sacchetti, B., Funari, A., Michienzi, S., *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**(2):324–336.

23 Horwitz, E.M., Le Blanc, K., Dominici, M., *et al.* Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 2005;**7**(5):393–395.

24 Bianco, P., Robey, P.G., and Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008;**2**(4): 313–319.

25 Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;**4**(5):267–274.

26 Zafonte, B.T., Liu, S., Lynch-Kattman, M., *et al.* Smad1 expands the hemangioblast population within a limited developmental window. *Blood* 2007;**109**(2):516–523.

27 Xie, J., Guo, L., Ruan, Y., *et al.* Laminarin-mediated targeting to Dectin-1 enhances antigen-specific immune responses. *Biochem Biophys Res Commun* 2010;**391**(1):958–962.

28 Cheng, J., Zhou, L., Qin, Y.S., *et al.* Donor apoptotic lymphocyte transfusion-induced liver allograft tolerance by up-regulation of $CD4^+CD25^+$regulatory T cells in peripheral blood. *Transplant Proc* 2009;**41**(9):3893–3897.

29 Shirasawa, S., Sekiya, I., Sakaguchi, Y., *et al. In vitro* chondrogenesis of human synovium-derived mesenchymal stem cells: optimal condition and comparison with bone marrow-derived cells. *J Cell Biochem* 2005,**97**(1):84–97.

30 Tropel, P., Noël, D., Platet, N., *et al.* Isolation and characterisation of mesenchymal stem cells from adult mouse bone marrow. *Exp Cell Res* 2004;**295**(2):395–406.

31 Javazon, E.H., Colter, D.C., Schwarz, E.J., and, Prockop, D.J. Rat marrow stromal cells are more sensitive to plating density and expand more rapidly from single-cell-derived colonies than human marrow stromal cells. *Stem Cells* 2001;**19**(3): 219–25.

32 Lennon, D.P., Edmison, J.M., and Caplan, A.I. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol* 2001;**187**(3):345–355.

33 Lunstrum, G.P., Keene, D.R., Weksler, N.B., *et al.* Chondrocyte differentiation in a rat mesenchymal cell line. *J Histochem Cytochem* 1999;**47**(1):1–6.

34 Pittenger, M.F., Mackay, A.M., Beck, S.C., *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411):143–147.

35 Barlow, S., Brooke, G., Chatterjee, K., *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* 2008;**17**(6):1095–1107.

36 Ilic, N., Brooke, G., Murray, P., *et al.* Manufacture of clinical grade human placenta-derived multipotent mesenchymal stromal cells. *Methods Mol Biol* 2011;**698**:89–106.

37 Phinney, D.G., Kopen, G., Isaacson, R.L., and Prockop, D.J. Plastic adherent stromal cells from the bone marrow of commonly used strains of inbred mice: variations in yield, growth, and differentiation. *J Cell Biochem* 1999;**72**(4):570–585.

38 Gronthos, S., Zannettino, A.C., Hay, S.J., *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* **116**(9):1827–1835.

39 Dominici, M., Le Blanc, K., Mueller, I., *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

40 Kern, S., Eichler, H., Stoeve, J., *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* **24**(5):1294–1301.

41 Da Silva Meirelles, L., Caplan, A.I., and Nardi, N.B. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 2008;**26**(9): 2287–2299.

42 Kuci, S., Kuçi, Z., Kreyenberg, H., *et al.* CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica* 2010;**95**(4):651–659.

43 Brooke, G., Tong, H., Levesque, J.P., and Atkinson, K. Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. *Stem Cells Dev* 2008;**17**(5):929–940.

44 Simmons, P.J. and Torok-Storb, B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* 1991;**78**(1):55–62.

45 Gieseke, F., Böhringer, J., Bussolari, R., *et al.* Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. *Blood* 2010;**116**(19):3770–3779.

46 Devine, S.M., Cobbs, C., Jennings, M., *et al.* Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003;**101**(8):2999–3001.

47 Jarocha, D., Lukasiewicz, E., and Majka, M. Advantage of mesenchymal stem cells (MSC) expansion directly from purified bone marrow $CD105^+$ and $CD271^+$ cells. *Folia Histochem Cytobiol* 2008;**46**(3):307–314.

48 Cordonnier, T., Langonné, A., Sohier, J., *et al.* Consistent osteoblastic differentiation of human mesenchymal stem cells with bone morphogenetic protein 4 and low serum. *Tissue Eng Part C Methods* 2011;**17**(3):249–259.

49 Prockop, D.J., Brenner, M., Fibbe, W.E., *et al.* Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;**12**(5):576–578.

50 Miura, M., Miura, Y., Padilla-Nash, H.M., *et al.* Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells* 2006;**24** (4):1095–1103.

51 Matigian, N., Brooke, G., Zaibak, F., *et al.* Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern. *Genom Data* 2015;**3**:70–74.

52 Weiss, M.L., Anderson, C., Medicetty, S., *et al.* Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* 2008;**26**(11):2865–2874.

53 Wang, M., Yang, Y., Yang, D., *et al.* The immunomodulatory activity of human umbilical cord blood-derived mesenchymal stem cells *in vitro*. *Immunology* 2009;**126**(2):220–232.

54 Le Blanc, K., Tammik, C., Rosendahl, K., *et al.* HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;**31**(10):890–896.

55 Majumdar, M.K., Keane-Moore, M., Buyaner, D., *et al.* Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* 2003;**10**(2):228–241.

56 Tse, W.T., Pendleton, J.D., Beyer, W.M., *et al.* Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003;**75**(3): 389–397.

57 Jiang, X.X., Zhang, Y., Liu, B., *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**(10):4120–4126.

58 Le Blanc, K., Tammik, L., Sundberg, B., *et al.* Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003;**57**(1):11–20.

59 Groh, M.E., Maitra, B., Szekely, E., and Koç, O.N. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol* 2005;**33**(8):928–934.

60 Di Nicola, M., Carlo-Stella, C., Magni, M., *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**(10): 3838–3843.

61 Aggarwal, S. and Pittenger, M.F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4): 1815–1822.

62 Jones, B.J., Brooke, G., Atkinson, K., and McTaggart, S.J. Immunosuppression by placental indoleamine 2,3-dioxygenase: a role for mesenchymal stem cells. *Placenta* 2007;**28**(11–12):1174–1181.

63 Chabannes, D., Hill, M., Merieau, E., *et al.* A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 2007;**110**(10):3691–3694.

64 Nasef, A., Mazurier, C., Bouchet, S., *et al.* Leukemia inhibitory factor: role in human mesenchymal stem cells mediated immunosuppression. *Cell Immunol* 2008;**253**(1–2):16–22.

65 Gieseke, F., Schütt, B., Viebahn, S., *et al.,* Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFNγR1 signaling and IDO expression. *Blood* 2007;**110**(6):2197–2200.

66 Selmani, Z., Naji, A., Zidi, I., *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**(1): 212–222.

67 Liotta, F., Angeli, R., Cosmi, L., *et al.* Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 2008;**26**(1):279–289.

68 Klyushnenkova, E., Mosca, J.D., Zernetkina, V., *et al.* T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* 2005;**12**(1):47–57.

69 Krampera, M., Cosmi, L., Angeli, R., *et al.* Role for IFN-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**(2):386–398.

70 Krampera, M., Glennie, S., Dyson, J., *et al.* Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;**101**(9): 3722–3729.

71 Benvenuto, F., Ferrari, S., Gerdoni, E., *et al.* Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* 2007;**25**(7):1753–1760.

72 Zhou, L., Chong, M.M., and Littman, D.R. Plasticity of $CD4^+$ T cell lineage differentiation. *Immunity* 2009;**30**(5):646–655.

73 Rafei, M., Campeau, P.M., Aguilar-Mahecha, A., *et al.* Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 2009;**182**(10):5994–6002.

74 Fox, J.M., Chamberlain, G., Ashton, B.A., and Middleton, J. Recent advances into the understanding of mesenchymal stem cell trafficking. *Br J Haematol* 2007;**137**(6):491–502.

75 Honczarenko, M., Le, Y., Swierkowski, M., *et al.* Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells* 2006;**24**(4):1030–1041.

76 Shi, M., Li, J., Liao, L., *et al.* Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. *Haematologica* 2007;**92**(7): 897–904.

77 Ponte, A.L., Marais, E., Gallay, N., *et al.* The *in vitro* migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 2007;**25**(7):1737–1745.

78 Wynn, R.F., Hart, C.A., Corradi-Perini, C., *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 2004;**104**(9):2643–2645.

79 Ceradini, D.J., Kulkarni, A.R., Callaghan, M.J., *et al.* Progenitor cell trafficking is regulated by hypoxic gradients through HIF-1 induction of SDF-1. *Nat Med* 2004;**10**(8):858–864.

80 Caplan, A.I. The mesengenic process. *Clin Plast Surg* 1994;**21**(3): 429–435.

81 Feng, X., Yang, H.R., Yu, S.Q., *et al.* Genetic analysis of the complete env genes of HIV-1 from paid blood donors in Henan province. *Bing Du Xue Bao* 2009;**25**(2):88–94(in Chinese).

82 Stenderup, K., Justesen, J., Clausen, C., and Kassem, M. Aging is associated with decreased maximal life span and accelerated senescence of bone marrow stromal cells. *Bone* 2003;**33**(6): 919–926.

83 Bergman, R.J., Gazi, D., Kahn, A.J., *et al.* Age-related changes in osteogenic stem cells in mice. *J Bone Miner Res* 1996;**11**(5):568–577.

84 Mosna, F., Sensebe, L., and Krampera, M. Human bone marrow and adipose tissue mesenchymal stem cells: a user's guide. *Stem Cells Dev* 2010;**19**(10):1449–1470.

85 Zhou, L.D., Zhang, Q.H., Zhang, Y., *et al.* The shiitake mushroom-derived immuno-stimulant lentinan protects against murine malaria blood-stage infection by evoking adaptive immune-responses. *Int Immunopharmacol* 2009;**9**(4):455–462.

86 Campagnoli, C., Roberts, I.A., Kumar, S., *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001;**98**(8):2396–2402.

87 Gu, X., Zhou, L., Du, Q., *et al.* Hesperetin inhibits the maturation and function of monocyte-derived dendritic cells from patients with asthma. *Mol Med Rep* 2009;**2**(3):509–513.

88 Ringe, J., Kaps, C., Burmester, G.R., and Sittinger, M. Stem cells for regenerative medicine: advances in the engineering of tissues and organs. *Naturwissenschaften* 2002;**89**(8):338–351.
89 Eibes, G., Debernardi, G., Feijoo, G., *et al.* Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase. *Biodegradation* 2011;**22**(3):539–550.
90 Brooke, G., Rossetti, T., Pelekanos, R., *et al.* Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. *Br J Haematol* 2009;**144**(4):571–579.
91 Crisan, M., Huard, J., Zheng, B., *et al.* Purification and culture of human blood vessel-associated progenitor cells. *Curr Protoc Stem Cell Biol* 2008;**2**:2B.2.1–2B.2.13.
92 Caplan, A.L. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009;**217**(2):318–324.
93 Bailo, M., Soncini, M., Vertua, E., *et al.* Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation* 2004;**78**(10):1439–1448.
94 Wulf, G.G., Viereck, V., Hemmerlein, B., *et al.* Mesengenic progenitor cells derived from human placenta. *Tissue Eng* 2004; **10**(7–8):1136–1147.
95 De Coppi, P., Bartsch Jr. G., Siddiqui, M.M., *et al.,* Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007;**25**(1):100–106.
96 In 't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., *et al.* Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 2003;**102**(4):1548–1549.
97 Tsai, M.S., Lee, J.L., Chang, Y.J., and Hwang, S.M. Isolation of human multipotent mesenchymal stem cells from second-trimester amniotic fluid using a novel two-stage culture protocol. *Hum Reprod* 2004;**19**(6):1450–1456.
98 Bieback, K., Kern, S., Klüter, H., and Eichler, H. Critical parameters for the isolation of mesenchymal stem cells from umbilical cord blood. *Stem Cells* 2004;**22**(4):625–634.
99 Romanov, Y.A., Svintsitskaya, V.A., and Smirnov, V.N. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells* 2003; **21**(1):105–110.
100 Sarugaser, R., Lickorish, D., Baksh, D., *et al.* Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* 2005;**23**(2):220–229.
101 Poloni, A., Rosini, V., Mondini, E., *et al.* Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta. *Cytotherapy* 2008;**10**(7):690–697.
102 In 't Anker, P.S., Noort, W.A., Scherjon, S.A., *et al.* Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* 2003;**88**(8):845–852.
103 Hu, Y., Liao, L., Wang, Q., *et al.,* Isolation and identification of mesenchymal stem cells from human fetal pancreas. *J Lab Clin Med* 2003;**141**(5):342–349.
104 Soncini, M., Vertua, E., Gibelli, L., *et al.,* Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* 2007;**1**(4):296–305.
105 Marcus, A.J. and Woodbury, D. Fetal stem cells from extra-embryonic tissues: do not discard. *J Cell Mol Med* 2008;**12**(3):730–742.
106 Shi, S., Robey, P.G., and Gronthos, S. Comparison of human dental pulp and bone marrow stromal stem cells by cDNA microarray analysis. *Bone* 2001;**29**(6):532–539.
107 Menicanin, D., Bartold, P.M., Zannettino, A.C., and Gronthos, S. Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. *Stem Cells Dev* **19**(10): 1501–1510.
108 Tsai, M.S., Hwang, S.M., Chen, K.D., *et al.* Functional network analysis of the transcriptomes of mesenchymal stem cells derived from amniotic fluid, amniotic membrane, cord blood, and bone marrow. *Stem Cells* 2007;**25**(10):2511–2523.
109 Weng, S.L., Chang, S.J., Cheng, Y.C., *et al.* Comparative transcriptome analysis reveals a fetal origin for mesenchymal stem cells and novel fetal surface antigens for noninvasive prenatal diagnosis. *Taiwan J Obstet Gynecol* 2011;**50**(4):447–457.
110 Miranda, H.C., Herai, R.H., Thomé, C.H., *et al.* A quantitative proteomic and transcriptomic comparison of human mesenchymal stem cells from bone marrow and umbilical cord vein. *Proteomics* 2012;**12**:2607–2617.
111 Spitzer, T.L., Rojas, A., Zelenko, Z., *et al.,* Perivascular human endometrial mesenchymal stem cells express pathways relevant to self-renewal, lineage specification, and functional phenotype. *Biol Reprod* 2012;**86**(2): *58*
112 Monaco, E., Bionaz, M., Rodriguez-Zas, S., *et al.* Transcriptomics comparison between porcine adipose and bone marrow mesenchymal stem cells during *in vitro* osteogenic and adipogenic differentiation. *PLoS ONE* 2012;**7**(3):e32481.
113 Lalu, M.M., McIntyre, L., Pugliese, C., *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.

CHAPTER 4

# The biology and clinical applications of mesenchymal stromal cells derived from human gestational tissues

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## 4.1 Introduction

The gestation period for humans is approximately 40 weeks with a normal range of 37–42 weeks. Placenta at this time is known as term placenta. The word placenta comes from a Greek word meaning "cake," referring to its round flat slab-like appearance (Figure 4.1).

The mammalian placenta is composed of both fetal and maternal elements. The chorion and amnion membranes on the outside of the placenta are of fetal origin. The rest of the placenta is of maternal origin. The human term placenta averages 22 cm (9 inch) in length and 2–2.5 cm (0.8–1 inch) in thickness, with the center being the thickest and the edges being the thinnest. It typically weighs approximately 500 g (just over 1 lb). It has a dark reddish–blue or crimson color. It connects to the fetus by the umbilical cord, which is approximately 55–60 cm (22–24 inch) in length and contains two umbilical arteries and one umbilical vein. The umbilical cord inserts into the chorionic plate. Vessels branch out over the surface of the placenta and further divide to form a network covered by a thin layer of cells. This results in the formation of villous tree structures. On the maternal side, these villous tree structures are grouped into lobules called cotyledons (Figure 4.2).

The human term placenta has aroused significant interest as a source of mesenchymal stromal cells (MSCs) for use in regenerative and immunodulatory medicine. Literally billions of MSCs can be isolated from a single placenta taken through to passage 5. It is an interesting tissue for studying MSCs because their environment is unique due to the development and growth of the fetus throughout gestation. The cells derived from the fetus are hypothesized to be more primitive than those derived from the mother due to the age and external environmental factors to which the mother may have been previously exposed, including pathogens or chemicals. Thus, we postulated that the MSCs that reside here may be more advantageous than the more traditional bone-marrow-derived MSCs for use in MSC-based clinical applications.

Term placental tissue is easily accessible, is usually considered a waste product after birth, and its use for treatment purposes is free from ethical concerns. Many reports have described stem/stromal cells from different components of the placenta using many different isolation and characterization techniques.

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**Figure 4.1** A human term placenta obtained at elective cesarean section after safe delivery of the baby. A clamp is seen clamping the umbilical cord on the top surface of the placenta.

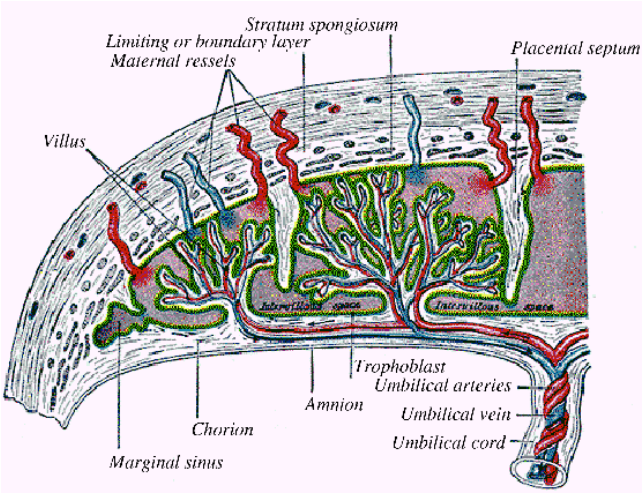



**Figure 4.2** The anatomy of the human placenta. Maternal blood fills the intervillous space, nutrients, water, and gases are actively and passively exchanged, and then deoxygenated blood is displaced by the next maternal pulse. *Source:* this image, reproduced from Wikimedia Commons, is in the public domain. Its attribution is "Gray39" by Henry Vandyke Carter – Henry Gray (1918) *Anatomy of the Human Body* (See "Book" section below) Bartleby.com: Gray's Anatomy, Plate 39. Licensed under Public Domain via Wikimedia Commons – http://commons.wikimedia.org/wiki/File:Gray39.png#/media/File:Gray39.png. (See color plate section.)

However, the lack of universal assays to define an MSC has resulted in many discrepancies in the literature.

Stem cells have two properties: they must be able to divide and be self-renewing, and they must be able to differentiate down a specific lineage or set of lineages to produce functional cells. In the 1960s, Alexander Friedenstein and colleagues in Russia discovered a type of adult stem cell that they called colony-forming units fibroblastic (CFU-Fs), since single cells grew into colonies and were readily distinguishable from hematopoietic stem cells (HSCs) by their adherence to plastic and their fibroblast-like morphology [1]. Friedenstein and Owen called these cells osteoprogenitors. Subsequently, these cells were shown to differentiate to key elements of the skeleton, including bone (osteoblasts), cartilage (chondrocytes), fat (adipocytes), and periosteum (fibroblasts). Evidence of self-renewal has been shown by Sacchetti and colleagues for osteoprogenitor cells in bone marrow sinusoids that were able to organize a hematopoietic microenvironment [2]. They showed that MCAM/CD146-expressing subendothelial cells in human bone marrow stroma were capable of transferring, upon transplantation, the hematopoietic microenvironment to heterotopic sites, coincident with the establishment of identical subendothelial cells within a miniature bone organ. Subsequently, Méndez-Ferrer and colleagues confirmed these findings by showing that mesenchymal and HSCs formed a unique bone marrow niche [3]. This area of science has been well summarized by Paolo Bianco and coworkers in 2008 [4], 2011 [5], and 2013 [6].

Thus, the term skeletal stem cell for these cells is well founded scientifically. True mesenchymal stem cells, however, are rare cells with a frequency in bone marrow of between 1 in $10^4$ and 1 in $10^5$. To enable both experimental and clinical trials of mesenchymal cells to be performed, it has been necessary to expand them in *in vitro* cultures. The cells obtained are not mesenchymal stem cells, but rather their progeny – often mistakenly called mesenchymal stem cells but in fact they are mesenchymal stromal or mesenchymal progenitor cells. In the rest of this chapter the term mesenchymal stromal cells will be used and the abbreviation MSCs will be applied only to mesenchymal stromal cells.

MSCs isolated from placental tissue (pMSCs) display phenotypic plasticity and are involved in maintaining fetal tolerance due to their immunomodulatory properties [7].

## 4.2 Isolation of placental mesenchymal stromal cells

Many studies have used different isolation and expansion techniques as well as different approaches to characterizing MSCs derived from human gestational products. This has made it difficult to compare and contrast different studies and their cell populations. To address this issue a report from an international workshop on placenta-derived stem/stromal cells published guidelines for the identification and verification of MSCs derived from placental tissues (pMSCs) in 2008 [7]. Minimal criteria for defining pMSCs, such as human amnion-derived MSCs (AMSCs) and human chorion-derived MSCs (CMSCs), indicated that such cells must be adherent to plastic, form fibroblast-like colony-forming units, express a characteristic pattern of surface antigens, and display differentiation potential toward one or more mesodermal lineages, including osteogenic, adipogenic, and chondrogenic lineages, and/or vascular/endothelial cell types. Furthermore, for cells to be deemed fetal in origin they had to demonstrate maternal contamination of 1% or less [7].

All but the last of these characteristics were similar to the criteria proposed by Dominici *et al.* in 2006 for adult bone-marrow-derived MSCs and adopted by the International Society

for Cell Therapy [8]. However, not all studies have reported pMSCs to possess these properties. In particular, there are conflicting reports from investigators regarding the origin of MSCs from term placental tissues. It has been reported that cultured populations of MSCs derived from placental fetal membranes consisted of cells of maternal origin presumed due to overgrowth of maternal MSCs that had "contaminated" the fetal tissues. Therefore, to document the frequency and determinants of maternal cell contamination in cultured chorionic MSCs, we undertook a PRISMA-compliant systematic review of publications in PubMed, Medline, and Embase databases from January 2000 till July 2013 on placental and/or chorionic MSCs from uncomplicated pregnancies [9]. We discovered that of 147 studies only 26 (18%) investigated the question of fetal and/or maternal cell origin. After excluding studies that did not satisfy minimal MSC criteria, we found 8 of 15 informative studies that documented MSC cultures as fetal in origin (53%). The remaining seven studies reported that expanded human chorionic MSC populations were either maternal in origin ($n = 6$) or mixed ($n = 1$). Maternal cell contamination was associated with term placentas, chorionic membrane samples, and greater passage number, but was also present in 3 of 10 studies of chorionic villous MSCs. While most studies have assumed a fetal origin for MSCs sourced from chorion, this systematic review documented a high incidence of maternal-origin MSC populations in pMSC cultures. Given that fetal MSCs may be more primitive than adult MSCs, our findings have implications for clinical trials. Therefore, we recommended sensitive methods to quantitate the source and purity of pMSCs [9].

Isolation of MSCs from whole term placenta involves dissection of the placenta into small pieces using scissors and scalpels followed by enzymatic digestion, removal of red cells by lysis or density gradient separation, and finally seeding cells from the mononuclear cell monolayer on to plastic tissue cultures flasks. The culture medium is a basic one such DMEM supplemented with 20% fetal calf serum (FCS). pMSCs generally become almost confluent after approximately 1 week in culture, when they are harvested and the next set of flasks are seeded.

## 4.3 Characteristics of fetally derived mesenchymal stromal cells isolated from gestational tissues

Fetally derived MSCs are theoretically attractive because they may contain less genetic abnormalities than adult-tissue-derived MSCs, have greater proliferative capacity, and a greater retention of "stemness" memory. It is thought that they have properties intermediate between embryonic and adult stem cells [10]. Thus, they may be a superior MSC source for clinical trials and therapies compared with traditional sources such as bone marrow.

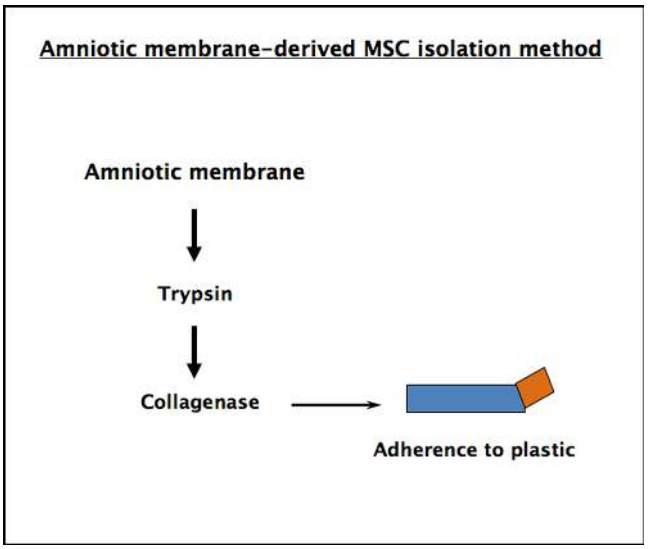



**Figure 4.3** The method used in our laboratory to isolate human AMSCs.

### 4.3.1 Amniotic-membrane-derived mesenchymal stromal cells

The amniotic membrane is a fetal tissue and contains a mesodermal layer in which MSCs reside. Many researchers have attempted to isolate fetally derived MSC-like cells from the amniotic membrane. Both mechanical and enzymatic digestions are amongst the most popular techniques.

There are many different methods used to isolate AMSCs. However, typically, AMSCs are isolated using trypsin followed by a collagenase digest [11] (Figure 4.3). AMSCs expanded in culture exhibit a fibroblast-like morphology and express CD90, CD105, and CD73 on their surface membrane but not CD45. AMSCs can differentiate *in vitro* along the mesodermal lineage, including osteocytes, chondrocytes, and in some studies adipocytes. Interestingly, AMSCs have been reported to differentiate into lineages of all three germ layers, ectoderm [12], mesoderm [13], and endoderm [14], and give rise to progeny such as lung alveolar type II cells, endothelial cells, hepatocytes, cardiomyocytes, and neuronal-like cells [15–18]. It has also been reported that AMSCs spontaneously differentiate *in vitro* into endothelial cells through secretion of vascular endothelial growth factor [15]. However, none of these studies showed any functional analysis of their findings *in vivo*, and this renders the claims to non-mesodermal lineage differentiation incomplete.

### 4.3.2 Chorionic-membrane-derived mesenchymal stromal cells

CMSCs are similar to AMSCs, in that they demonstrate mesodermal lineage differentiation potential *in vitro*, do not form teratomas *in vivo*, and have an immune privileged status [19,20]. Despite CMSCs residing in a separate niche to AMSCs, they are still fetus derived from the extra-embryonic blastocyst.

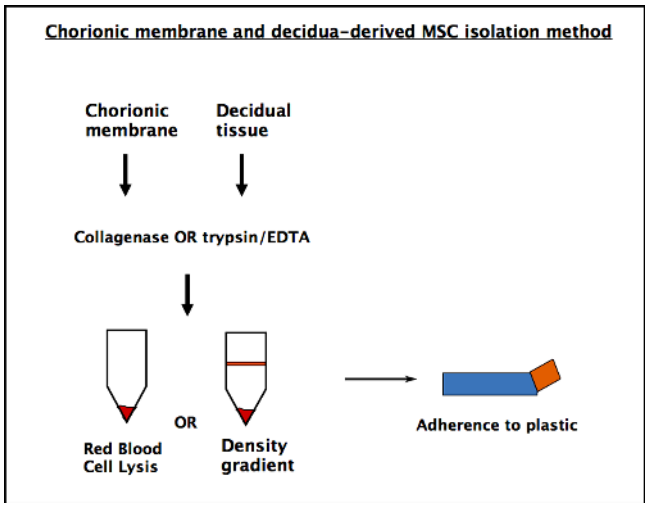



**Figure 4.4** The method used in our laboratory to isolate CMSCs and decidua MSCs.

Numerous methods have been adapted to isolate these cells, including mechanical digestion, explant cultures, and enzymatic digestion, similar to those used for AMSCs. To obtain potentially purified CMSC populations, the chorionic membrane must be physically separated from the rest of the placenta. This is usually followed by enzymatic digestion, such as collagenase or trypsin/EDTA digestion, followed further by either red blood cell lysis or a Ficoll density gradient to remove debris and red blood cells. The mononuclear layer of cells containing MSCs is then collected and plated in culture, where they usually adhere within 1 day (Figure 4.4). Contaminating nonadherent cells, such as hematopoietic cells, are washed and removed from culture 48–72 h after plating.

The chorionic villi of human term placenta are also a rich source of MSCs. Many studies have reported the isolation of CMSCs from the chorionic villi [7,19,21–26]. To remove contaminating decidua tissue of maternal origin, the villi are carefully separated from the decidua using a dissection microscope and then the villous tissue is enzymatically and mechanically digested prior to expansion in culture. It is thought that the stem cell niche within the chorionic villi regulates the way CMSCs participate in placental tissue generation, maintenance, and repair. A number of cell surface markers have been used to identify the stem cell niche within the chorionic villi of first trimester and term human placenta. These have included pericyte cell surface markers such as CD146 and other markers such as STRO-1 and microtubule-associated protein 1B (MAP1B) which have been used to identify MSCs in other tissues [27]. One study indicated that some CMSCs have a pericyte-like phenotype. Furthermore, it has now been proposed that the vascular niche harbors a pool of MSCs that can give rise to committed progenitors for tissue maintenance and repair and that these MSCs contribute to vessel maturation and stabilization [28].

## 4.4 Characteristics of maternally derived mesenchymal stromal cells isolated from gestational tissue (the decidua)

Guo *et al.* described an adult stem/stromal cell population reported to have remarkable regeneration ability [29]. This population of cells was found within the decidua tissue and was thought to be responsible for the implantation of the embryo and formation of the placenta in the uterus. There is limited knowledge regarding decidua-derived MSCs. Our laboratory found that MSCs isolated from whole placentas were of maternal origin despite the babies being of male gender [30,31]. This indicated maternal MSC contamination and subsequent overgrowth of maternal MSCs in these placental cultures [30–33]. Because of these findings we undertook a study to determine a method for isolating and characterizing fetal-derived cells from term placenta uncontaminated by maternal cells.

## 4.5 Comparison of mesenchymal stromal cells from fetal and maternal tissues isolated from gestational tissues

We established a method for isolating efficient and viable amnion, chorion, and decidua MSC populations [34] (Figures 4.3 and 4.4). Using placentas from babies of male gender we found CMSCs to be of male origin throughout their culture period, while AMSCs were initially of male origin but were subsequently overgrown in culture by MSCs of maternal origin. MSCs derived from decidua were always of female origin. We therefore focused on searching for differences between CMSCs and decidua-derived MSCs [34]. MSC cultures were characterized by cell morphology, cell surface phenotype, clonogenic ability, proliferative capacity, mesodermal differentiation ability, immune suppressive capacity, genotyping, as well as fluorescence *in situ* hybridization or sex-determining region of the Y chromosome analysis to determine the gender of the source of the cells [34]. All three cell populations (amnion, chorion, and decidua) displayed similar cell morphology and characteristic MSC cell surface marker expression. However, some differences were noted between the three populations. Cell proliferation differences were found between all three populations in early passages. Decidua MSCs had a more than twofold increase in population doublings compared with that of AMSC and CMSC populations in early cultures, and this likely explained the maternal overgrowth in MSC populations derived from the chorion [34].

Other differences were noted, such as the limited adipogenic potential of gestational product MSCs compared with that of bone marrow MSCs [34]. These findings re-enforced the concept that multipotent stroma-like cells from different tissues are similar but not identical [35]. Although amnion and decidua MSCs showed some similarities, they were found to be unique cell populations. We compared the gene expression profiles of

each population of fetal- and maternal-derived MSCs in order to further explore differences between these MSC populations [34].

The top functional pathways for AMSCs involved cell-to-cell signaling, cell death, and the development and function of the hematological system. These pathways were regulated by the transcription factors NFkB and SOX9, which are known to regulate cellular and organismal processes such as immune and inflammatory responses, developmental processes, cellular growth, and apoptosis. Several AMSC molecules were of interest, including CD46, a complement regulatory protein, and NIPA-like domain containing 3 (NIPAL3). Although CD46 is not a unique MSC marker, it could be used in conjunction with characteristic MSC markers to discriminate between fetal and maternal MSC populations. NIPAL3 was also a molecule of interest since little is known about its function. Therefore, together these molecules could be used to enrich for a fetal MSC population. NFkB and SOX9 may play a critical role in AMSC survival and immune response, and it is proposed that AMSCs are involved in the innate immune response against viruses and bacteria, representing at least part of the neonatal defense system against the earliest encountered pathogens [34]. Tsai *et al.* reported similar findings in term cord blood MSCs [36].

In contrast, the top functional pathways of decidua MSCs were cellular development and growth and proliferation [34]. These results are compatible with the finding that decidua MSCs proliferate faster and outgrow fetal MSCs in culture, indicating more robust self-renewal and differentiation potential. It was also found that numerous transcription factors, including *FOXC1*, *FOXF1*, *FOXL2*, *FOXO2*, *GATA2*, *HAND2* and *HOX* genes, were upregulated in the decidua MSC population [34]. This has also been described in endometrial stromal cell populations, as well as in embryonic stem cells and HSCs.

The progesterone receptor was also enriched in the decidua MSC population. This may indicate that decidua MSCs are regulated by progesterone. Moreover, two zinc finger proteins, zinc finger MIZ-type containing 1 and zinc finger protein 318, with no known function were also upregulated. This indicated that they are involved in MSC development, growth, and proliferation [34]. It was also found that many plasma membrane molecules were upregulated in the decidua MSC population. The G-protein receptor CD97 was upregulated and may prove to be a specific decidua MSC marker. It was also found that endothelin, bradykinin receptor B1, and thrombin receptors were expressed [34]. These genes were also reported to be upregulated in MSCs from amniotic fluid and amniotic-membrane-derived MSCs in the study by Tsai *et al.* This may indicate that they are involved in uterine contractions, and Tsai *et al.* considered that they may have a role in the homeostasis of fluids and electrolytes.

Functional network analyses in this study provided insights into the transcriptomes of MSCs derived from different sources [34]. AMSCs were involved in cell death and hematological system development and function, and, thus, as indicated earlier, may be involved in the neonatal innate immune system. Decidua MSCs were involved in cell development, growth, and proliferation, characteristic of their tissue of origin – the highly proliferative and regenerative endometrium. Molecules were identified at the transcriptomic level that may discriminate between fetal and maternal populations. However, future studies are required to confirm these findings at the protein level.

AMSCs did not proliferate as efficiently as their maternal counterpart [34]. Therefore, for pragmatic reasons, such as obtaining sufficient MSCs for clinical trials, large-scale expansion of decidual MSCs is our current preferred source for MSCs. Decidua MSCs may prove most useful in their own environment; for example, in improving the implantation rate of the blastocyst during *in vitro* fertilization treatments: a phase 1 clinical trial has been registered to investigate this (clinicaltrials.gov identifier: NCT01649752).

## 4.6 Comparison of gene expression profiles between human term-placenta-derived mesenchymal stromal cells, human adult bone-marrow-derived mesenchymal stromal cells, and human umbilical-cord-derived unrestricted somatic stem cells

We compared gene expression profiles by microarray of these three populations with cells from each source derived from four different donors. Principle component analysis was performed to demonstrate the difference between the cell sources and the reproducibility of the replicate donors. When plotting on the first two components, the samples clustered based on their tissue source, but some donor variation was apparent (Figure 4.5a). All replicates were tightly clustered except for one unrestricted somatic stem cell (USSC) sample (Chip ID: 4294077038_D; Sample ID: USSC4). However, this sample was still highly correlated to the other USSC donors (average Pearson of 0.89) compared with 0.95 for the bone marrow MSCs and the pMSCs. When plotting components 2 and 3 the samples clustered based on their tissue source (Figure 4.5b).

## 4.7 Preclinical mesenchymal stromal cell studies

Several animal studies have demonstrated prolonged survival of human AMSCs with no evidence of immunological rejection after xenogeneic transplantation into immune competent animals, including rats [38,39] and swine [38]. Additionally, human amniotic fluid MSCs appeared to be relatively resistant to rejection by the recipient, possibly due to their expression of immunosuppressive factors such as CD59 (protectin) and HLA-G [40]. Cotransplantation of umbilical cord blood HSCs together with fetal MSCs can reduce the risk of graft-versus-host disease in HSC transplant recipients, as well as enhance umbilical cord

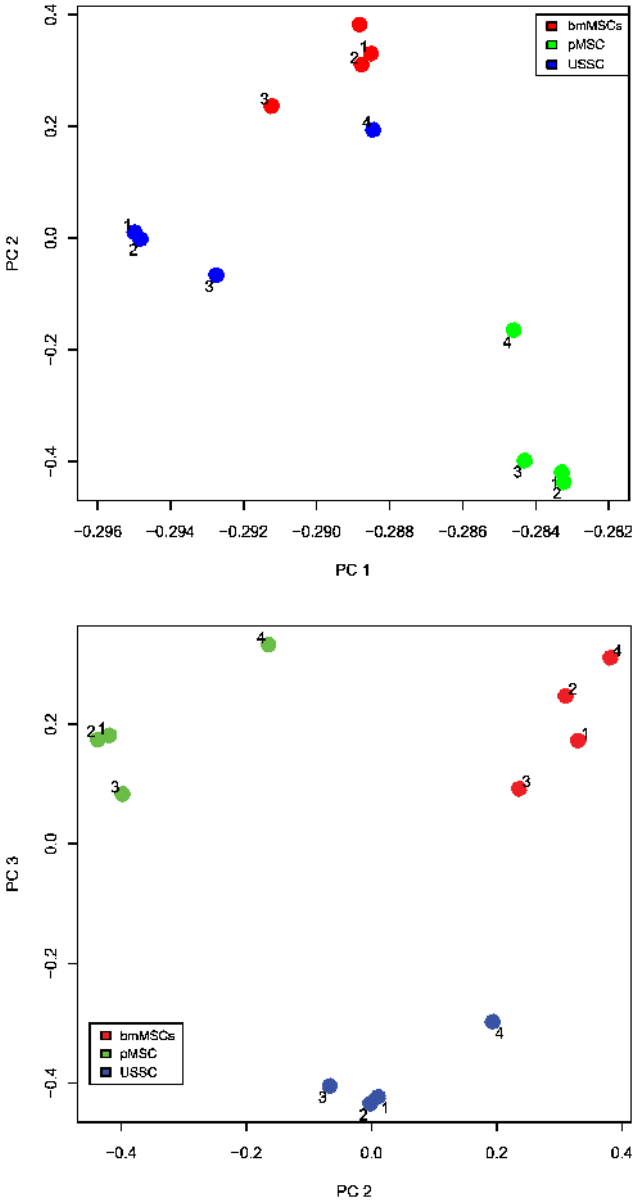



**Figure 4.5** Quality control and principal component analysis of gene expression data (detected probes only). (a) Comparing components 1 (89.1% variance) and 2 (4.1% variance); (b) Comparing components 2 (4.1% variance) and 3 (2.3% variance). *Source:* the images are reproduced from [37], Matigian *et al.*, *Genomics Data* 2015; **3**, 70–74 under a Creative Commons License (CC BY). doi:10.1016/j.gdata.2014.11.011.

blood cell engraftment and the homing of $CD34^+$ HSCs [41,42]. Amniotic membrane MSCs have been reported to promote the repair of ischemic heart damage [43]. Transplantation of AMSCs efficiently improved left ventricular function and reduced scar tissue in the myocardium after acute myocardial infarction in a rat model. AMSCs have also been reported to be neuroprotective in animal models of Parkinson's disease and stroke [39,44]. Transplantation of allogeneic and xenogeneic placenta-derived cells reduced bleomycin-induced lung fibrosis in mice [45] and proved effective in mice with critical limb ischemia [46].

Preclinical studies in mice with osteogenesis imperfecta showed that first-trimester fetal blood MSCs markedly reduced fracture rates and skeletal abnormalities [47]. Firinci *et al.* examined the efficacy of MSCs on lung histopathology in a murine model of chronic asthma. The results showed that MSCs migrated to lung tissue and ameliorated allergic asthma [48].

## 4.8 Clinical applications of placental mesenchymal stromal cells

MSCs are being tested in clinical trials, with over 450 MSC-related clinical trials listed in www.clinicaltrials.gov. There are currently 194 registered bone marrow MSC clinical trials and 28 pMSC trials registered. A meta-analysis of clinical trials in which MSCs were administered either intravenously or intra-arterially to over 1000 patients, over 300 of whom were recruited in randomized clinical trials, did not report a single serious adverse event associated with administration of the MSCs [49].

National regulatory bodies provide guidelines on the administration of cellular products for human use including requirements for current good manufacturing practice. Release criteria for the administration of MSCs in clinical trials should include cell viability, purity, sterility, negative mycoplasma testing, and minimal levels of endotoxin [50]; and see Chapters 43 and 44.

Most protocols for the manufacture of clinical-grade MSCs use FCS as a component of the *ex vivo* expansion medium. This is currently approved by the Australian regulatory authority, the Therapeutics Goods Authority as long as the FCS is sourced from a herd in a country that has never had a case of bovine spongiform encephalopathy (mad cow disease – http://www.tga.gov.au/industry/clinical-trials-handbook.htm). This is likely to change in the future when a serum-free medium will likely be required. It will be important to determine MSC genotype, phenotype, and function when culture media are modified.

Since allogeneic MSCs appear as effective as autologous MSCs, they can be manufactured in bulk and cryopreserved until needed. They are thus an off-the-shelf therapeutic agent similar to conventional therapeutic drugs. It is likely that MSC banks will be established. After several passages of *ex vivo* expansion, MSCs can be cryopreserved and stored long term, shipped to a clinical site, and thawed without loss of function. Haack-Sorensen *et al.* compared fresh and frozen *ex vivo* expanded MSCs and found that frozen MSCs still expressed the same phenotypic and cellular characteristics as fresh MSCs [51]; and see Chapter 7.

## 4.9 Manufacturing clinical-grade placenta-derived mesenchymal stromal cells

Our laboratory has provided maternal-derived placental MSCs for clinical trials [32]. Manual manufacturing of MSCs in large

quantities is laborious, time consuming, and expensive. An automated closed system would be highly beneficial. Pluristem Therapeutics Inc., an Israeli company, has developed large-scale production of placenta-derived MSCs using their proprietary PluriXTM 3D bioreactor system. Using this system, they have been able to expand adherent pMSCs (PLX-PAD cells) that retained their immunomodulatory properties [52]. A closed system also decreases the potential risk of contaminating the cell product from external environmental factors.

### 4.9.1 Phase 1 clinical trials using unrelated major-histocompatibility-unmatched placenta-derived mesenchymal stromal cells

Our laboratory generated enough maternally derived placental MSCs for three phase 1 clinical trials using two maternal donors who were unrelated to, and major histocompatibility (MHC) unmatched with, the MSC recipients. Our first trial was a phase 1 multicenter open-label dose-escalation study of unrelated MHC-unmatched placenta-derived mesenchymal stromal cells in recipients of unrelated umbilical cord blood hematopoietic stem cell transplants. No adverse events related to the MSCs were reported [50].

The second trial was a phase 1 trial of unrelated MHC-unmatched placenta-derived MSCs administered intravenously in patients with idiopathic pulmonary fibrosis. This disease affects middle-aged individuals and there is no effective treatment other than lung transplantation. It is usually fatal after 3–5 years. The primary objective of this trial was to establish the feasibility and safety of intravenous infusions of pMSCs in people with this serious lung disease. No serious adverse events related to the MSCs were noted [53].

The third trial was a phase 1 trial of pMSCs administered intratendinously in recipients with treatment-refractory Achilles tendinopathy. Two of the three planned dose escalation cohorts with MSCs injected directly into the tendon under ultrasound guidance were performed. No serious adverse events related to the MSCs were noted and a remarkable improvement in symptoms and exercise performance was noted (see Chapter 47).

## 4.10 Conclusions

At term the placenta contains both fetal and maternal MSC populations. The amniotic membrane is fetal in origin and is the preferred source for isolating human term fetal MSCs from the human term gestational product. The decidua is maternal in origin and is the preferred source for isolating maternally derived MSCs. Both of these MSC populations exhibited unique cellular and molecular characteristics and functional abilities reflective of their environment. Owing to the higher proliferation potential of maternal MSCs over fetal MSCs, they are at present the preferred gestational product MSC for large-scale production in our clinical trials program.

## References


1 Friedenstein, A.J., Piatetzky, I.I., and Petrakova, K.V. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966; **16**:381–390.

2 Sacchetti, B., Funari, A., Michienzi, S., *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–336.

3 Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

4 Bianco, P., Robey, P.G., and Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts and assays. *Cell Stem Cell* 2008;**2**:313–319.

5 Bianco, P. Back to the future: moving beyond "mesenchymal stem cells". *J Cell Biochem* 2011;**112**:1713–1721.

6 Bianco, P., Cu, X., Frenette, P.S., *et al.* The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;**19**:35–42.

7 Parolini, O., Alviano, F., Bagnara, G.P., *et al.* Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008;**26**(2):300–311.

8 Dominici, M., Le Blanc, K., Mueller, I., *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

9 Heazlewood, C.F., Sherrell, H., Ryan, J., *et al.* High incidence of contaminating maternal cell overgrowth in human placental mesenchymal stem/stromal cell cultures: a systematic review. *Stem Cells Transl Med* 2014;**3**(11):1305–1311.

10 Guillot, P.V., O'Donoghue, K., Kurata, H., and Fisk, N.M. Fetal stem cells: betwixt and between. *Semin Reprod Med* 2006; **24**(5):340–347.

11 Whittle, W.L., Gibb, W., and Challis, J.R. The characterization of human amnion epithelial and mesenchymal cells: the cellular expression, activity and glucocorticoid regulation of prostaglandin output. *Placenta* 2000;**21**(4):394–401.

12 Portmann-Lanz, C.B., Schoeberlein, A., Huber, A., *et al.* Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. *Am J Obstet Gynecol* 2006;**194**(3): 664–673.

13 Diaz-Prado, S., Muiños-López, E., Hermida-Gómez, T., *et al.* Human amniotic membrane as an alternative source of stem cells for regenerative medicine. *Differentiation* 2011;**81**(3):162–171.

14 Saulnier, N., Lattanzi, W., Puglisi, M.A., *et al.* Mesenchymal stromal cells multipotency and plasticity: induction toward the hepatic lineage. *Eur Rev Med Pharmacol Sci* 2009;**13**(Suppl 1):71–78.

15 Alviano, F., Fossati, V., Marchionni, C., *et al.* Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells *in vitro*. *BMC Dev Biol* 2007;**7**:11.

16 Zhao, P., Ise, H., Hongo, M., *et al.* Human amniotic mesenchymal cells have some characteristics of cardiomyocytes. *Transplantation* 2005;**79**(5):528–535.

17 Macias, M.I., Grande, J., Moreno, A., *et al.* Isolation and characterization of true mesenchymal stem cells derived from human term decidua capable of multilineage differentiation into all 3 embryonic layers. *Am J Obstet Gynecol* 2010;**203**(5):495.e9–495.e23.

18 Miki, T., Marongiu, F., Ellis, E.C., *et al.* Production of hepatocyte-like cells from human amnion. *Methods Mol Biol* 2009;**481**:155–168.

19 Soncini, M., Vertua, E., Gibelli, L., *et al.* Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* 2007;**1**(4):296–305.

20 Bacenkova, D., Rosocha, J., Tóthová, T., *et al.* Isolation and basic characterization of human term amnion and chorion mesenchymal stromal cells. *Cytotherapy* 2011;**13**(9):1047–1056.

21 Poloni, A., Rosini, V., Mondini, E., *et al.* Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta. *Cytotherapy* 2008;**10**(7):690–697.

22 Battula, V.L., Bareiss, P.M., Treml, S., *et al.* Human placenta and bone marrow derived MSC cultured in serum-free, b-FGF-containing medium express cell surface frizzled-9 and SSEA-4 and give rise to multilineage differentiation. *Differentiation* 2007;**75**(4):279–291.

23 Chen, M., Ng, L., Lau, E., *et al.* The effect of volume of chorionic villi on long-term cell culture. *Fetal Diagn Ther* 2008;**24**(4):409–412.

24 Fukuchi, Y., Nakajima, H., Sugiyama, D., *et al.* Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 2004;**22**(5):649–658.

25 Igura, K., Zhang, X., Takahashi, K., *et al.* Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy* 2004;**6**(6):543–553.

26 Zhang, X., Mitsuru, A., Igura, K., *et al.* Mesenchymal progenitor cells derived from chorionic villi of human placenta for cartilage tissue engineering. *Biochem Biophys Res Commun* 2006;**340**(3):944–952.

27 Crisan, M., Yap, S., Casteilla, L., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

28 Castrechini, N.M., Murthi, P., Gude, N.M., *et al.* Mesenchymal stem cells in human placental chorionic villi reside in a vascular niche. *Placenta* 2010;**31**(3):203–212.

29 Guo, C., Zhu, H., Huang, W., *et al.* Side population cells in the human decidua of early pregnancy exhibit stem/progenitor cell-like characteristics. *Reprod Biomed Online* 2010;**21**(6):783–793.

30 Barlow, S., Brooke, G., Chatterjee, K., *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* 2008;**17**(6):1095–1107.

31 Semenov, O.V., Koestenbauer, S., Riegel, M., *et al.* Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. *Am J Obstet Gynecol* 2010;**202**(2):193.e1–193.e13.

32 Brooke, G., Rossetti, T., Pelekanos, R., *et al.* Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. *Br J Haematol* 2009;**144**(4):571–579.

33 Strakova, Z., Livak, M., Krezalek, M., and Ihnatovych, I. Multipotent properties of myofibroblast cells derived from human placenta. *Cell Tissue Res* 2008;**332**(3):479–488.

34 Heazlewood, C.F. A comparative study of fetal and maternal derived mesenchymal stromal cells from the term placenta for cellular therapy. The University of Queensland, PhD Thesis, 2013.

35 Pelekanos, R.A., Li, J., Gongora, M., *et al.* Comprehensive transcriptome and immunophenotype analysis of renal and cardiac MSC-like populations supports strong congruence with bone marrow MSC despite maintenance of distinct identities. *Stem Cell Res* 2012;**8**(1):58–73.

36 Tsai, M.S., Hwang, S.M., Chen, K.D., *et al.* Functional network analysis of the transcriptomes of mesenchymal stem cells derived from amniotic fluid, amniotic membrane, cord blood, and bone marrow. *Stem Cells* 2007;**25**(10):2511–2523.

37 Matigian, N., Brooke, G., Zaibak, F., *et al.* Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern. *Genom Data* 2015;**3**:70–74.

38 Bailo, M., Soncini, M., Vertua, E., *et al.* Engraftment potential of human amnion and chorion cells derived from term placenta. *Transplantation* 2004;**78**(10):1439–1448.

39 Kong, X.Y., Cai, Z., Pan, L., *et al.* Transplantation of human amniotic cells exerts neuroprotection in MPTP-induced Parkinson disease mice. *Brain Res* 2008;**1205**:108–115.

40 Walther, G., Gekas, J., and Bertrand, O.F. Amniotic stem cells for cellular cardiomyoplasty: promises and premises. *Catheter Cardiovasc Interv* 2009;**73**(7):917–924.

41 In 't Anker, P.S., Scherjon, S.A., Kleijburg-van der Keur, C., *et al.* Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 2003;**102**(4):1548–1549.

42 Hiwase, S.D., Dyson, P.G., To, L.B., and Lewis, I.D. Cotransplantation of placental mesenchymal stromal cells enhances single and double cord blood engraftment in nonobese diabetic/severe combined immune deficient mice. *Stem Cells* 2009;**27**(9):2293–2300.

43 Fujimoto, K.L., Miki, T., Liu, L.J., *et al.* Naive rat amnion-derived cell transplantation improved left ventricular function and reduced myocardial scar of postinfarcted heart. *Cell Transplant* 2009;**18**(4):477–486.

44 Yu, S.J., Soncini, M., Kaneko, Y., *et al.* Amnion: a potent graft source for cell therapy in stroke. *Cell Transplant* 2009;**18**(2):111–118.

45 Cargnoni, A., Gibelli, L., Tosini, A., *et al.* Transplantation of allogeneic and xenogeneic placenta-derived cells reduces bleomycin-induced lung fibrosis. *Cell Transplant* 2009;**18**(4):405–422.

46 Prather, W.R., Toren, A., Meiron, M., *et al.* The role of placental-derived adherent stromal cell (PLX-PAD) in the treatment of critical limb ischemia. *Cytotherapy* 2009;**11**(4):427–434.

47 Guillot, P.V., Abass, O., Bassett, J.H., *et al.* Intrauterine transplantation of human fetal mesenchymal stem cells from first-trimester blood repairs bone and reduces fractures in osteogenesis imperfecta mice. *Blood* 2008;**111**(3):1717–1725.

48 Firinci, F., Karaman, M., Baran, Y., *et al.* Mesenchymal stem cells ameliorate the histopathological changes in a murine model of chronic asthma. *Int Immunopharmacol* 2011;**11**(8):1120–1126.

49 Lalu, M.M., McIntyre, L., Pugliese, C., *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.

50 Brooke, G., Rossetti, T., Ilic, N., *et al.* Points to consider in designing mesenchymal stem cell-based clinical trials. *Transfus Med Hemother* 2008;**35**(4):279–285.

51 Haack-Sorensen, M., Bindslev, L., Mortensen, S., Friis, T., and Kastrup, J. The influence of freezing and storage on the characteristics and functions of human mesenchymal stromal cells isolated for clinical use. *Cytotherapy* 2007;**9**(4):328–337.

52 Prather, W. Pluristem Therapeutics, Inc. *Regen Med* 2008;**3**(1):117–122.

53 Chambers, D.C., Enever, D., Ilic, N., *et al.* A phase 1b study of placenta-derived mesenchymal stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2004;**19**(7):1013–1018.

CHAPTER 5

# Human placenta-derived mesenchymal stem/stromal cells: fetal and maternal origins and critical parameters for *ex vivo* expansion

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## 5.1 Introduction

The placenta is derived from the blastocyst and composed of fetal cells, although it is intimately linked to the maternal circulation and uterine lining throughout pregnancy [1,2]. Even after delivery the placental tissue contains both fetal and maternal cells. The human placenta and adnexa are a rich source of several stem and progenitor cells types, including mesenchymal stem/stromal cells (MSCs), endothelial progenitor cells (EPCs), and hematopoietic stem cells (HSCs) [3–6]. Research into placental-derived cells is increasing due to their tissue regeneration potential for a number of human diseases [7].

However, full understanding of the basic biology of placenta-derived cells, in particular its MSCs, is lacking. The literature reports that both fetal and maternal MSCs can be cultured *ex vivo* from the placental chorion. A recent systematic review of the literature of 147 papers that isolated chorionic MSCs found that only 15 determined if the cells were fetal or maternal in origin [8]. Of those papers examining donor origin, half reported the cells were of fetal origin and half reported the cells were of maternal or mixed origin. None of the papers indicated clearly why fetal or maternal cells were obtained, nor did the systematic review shed light on to procedural conditions that could have been responsible for the differences in MSC origin.

This chapter discusses our approach to isolating fetal or maternal MSCs from the chorionic villi (CV) of the human term placenta. We discuss the conditions required for fetal MSC proliferation, prevention of maternal MSC contamination, and determination of the optimal method to quantitate fetal/maternal MSCs in culture.

## 5.2 Mesenchymal stem/stromal cells: a consensus definition?

MSCs from both adult and perinatal sources have been isolated [9–18] and are often characterized using the guidelines proposed by the International Society for Cellular Therapy (ISCT) [19]. According to these guidelines, adult bone marrow MSCs (BM-MSCs) are defined as (1) cells that possess a

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fibroblast-like morphology, (2) are adherent to plastic, (3) differentiate *in vitro* to cells of mesodermal lineages, (4) express the cell surface markers CD105, CD73, and CD90, and (5) do not express hematopoietic or endothelial markers such as CD34 and CD31. Although this definition was initially developed for adult bone-marrow-derived MSCs it has since been applied to human and non-human MSCs from different organs [9,20–22]. However, more recent literature suggests that MSCs should be characterized based on their tissue source and/or potential clinical application, as the ISCT definition did not address these issues [23,24]. One significant advance was made in 2013 in a joint statement from the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy on stromal cells from the adipose-tissue-derived stromal vascular fraction and culture expanded adipose-tissue-derived stromal/stem cells (ADSCs). The centrifuged but uncultured stromal vascular fraction was identified as being $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$ (platelet endothelial cell adhesion molecule), and $CD34^+$, while cell surface positivity for CD13 (alanine aminopeptidase), CD73, CD90, and CD105 are best used in conjunction with CD45, CD235a, and CD31. *Ex-vivo*-expanded ADSCs retain cell surface markers expressed by other MSCs, including CD44 (the hyaluronic acid receptor), CD73, CD90, and CD105, and are also negative for CD45 and CD31, but can be distinguished from bone-marrow-derived MSCs by being $CD36^+$ (fatty acid translocase, also known as glycoprotein IIIb) and $CD106^-$ (vascular cell-adhesion molecule 1) (and see Chapter 1).

To date, no one cell surface marker has been identified that is specific to MSCs or is able to prospectively isolate MSCs *ex vivo* [25,26], although the antibodies Stro-1 and Stro-3 have been used to obtain a purified population of MSCs from adult bone marrow. Studies have suggested that factors including donor age, cell source, and niche are responsible for variability in basic characteristics such as differentiation propensities and cell surface markers [23]. Additionally, *in vitro* culture-expanded MSCs are a heterogeneous population and may comprise a mixture of true stem cells with fully and partially committed mesodermal progenitors such as osteoblasts, chondrocytes, and adipocytes [26]. Notably, MSCs differ in their differentiation and proliferation characteristics depending on their tissue of origin [27–29].

## 5.3 Prenatal and perinatal tissue sources of mesenchymal stem/stromal cells

MSCs derived from prenatal and perinatal tissues, including the fetus, placenta, umbilical cord, and amniotic fluid, have the basic properties of all MSCs. However, they also are reported to possess a wider differentiation potential, greater colony forming ability, faster growth kinetics, and greater long-term proliferative ability in *ex vivo* expansion cultures than adult MSCs [30–32]. It is emerging that, when directly compared, MSCs from different origins often show subtle differences. Because of their primitive characteristics and ease of isolation prenatal and perinatal MSCs offer promising alternatives as a source of MSCs for clinical trials.

## 5.4 Fetal tissue-derived mesenchymal stem/stromal cells

MSCs have been isolated and characterized from bone marrow, blood, and liver from the early trimester human fetus [30]. Fetal BM-MSCs were determined to have an increased osteogenic capacity in *vitro* and *in vivo* [31–33], greater expansion capacity and faster doubling time, a greater telomere length, higher telomerase activity, and greater telomerase reverse transcriptase gene expression than adult BM-MSCs [13,32]. Although fetal tissue MSCs may appear to be superior to adult MSCs [34], associated ethical issues and the small amount of starting material are disadvantages.

## 5.5 Placental and adnexal stem and progenitor cells

Stem and progenitor cells have been derived from amniotic fluid [35–37], umbilical cord [38], and first-, second-, and third-trimester placenta, including the amniotic membrane, chorionic membrane, chorionic plate, CV, and maternal decidua [12,17,39–41] (Figure 5.1).

The derived stem/progenitor cells include amniotic fluid stem cells [35], EPCs [42,43], chorionic trophoblastic cells, amniotic epithelial cells [41], and MSCs [41,44]. MSCs isolated from these anatomical locations have been reported to have different properties. For example, MSCs isolated from the villi/decidua proliferate faster than amniotic membrane-derived MSCs (AM-MSCs) [41], with decidual MSCs (D-MSCs) demonstrating the highest proliferation rate in some studies [44,45]. Additionally, MSCs isolated from different regions vary in their fetal or maternal origin. For example, maternal MSCs often contaminate fetal MSCs isolated from the CV [17,46–48]. Compared with adult BM-MSCs, placental MSCs have greater proliferative capacity and immunomodulatory potential and are generally reported to be equivalent in terms of their osteogenic and chondrogenic differentiation *in vitro*, but they have a lower adipogenic differentiation potential [17,46].

## 5.6 Comparison of mesenchymal stem/stromal cells from different gestational sources

Interestingly, MSCs from the same placenta have different functional properties depending on their anatomical origin. CV-MSCs had greater osteogenic potential than AM-MSCs

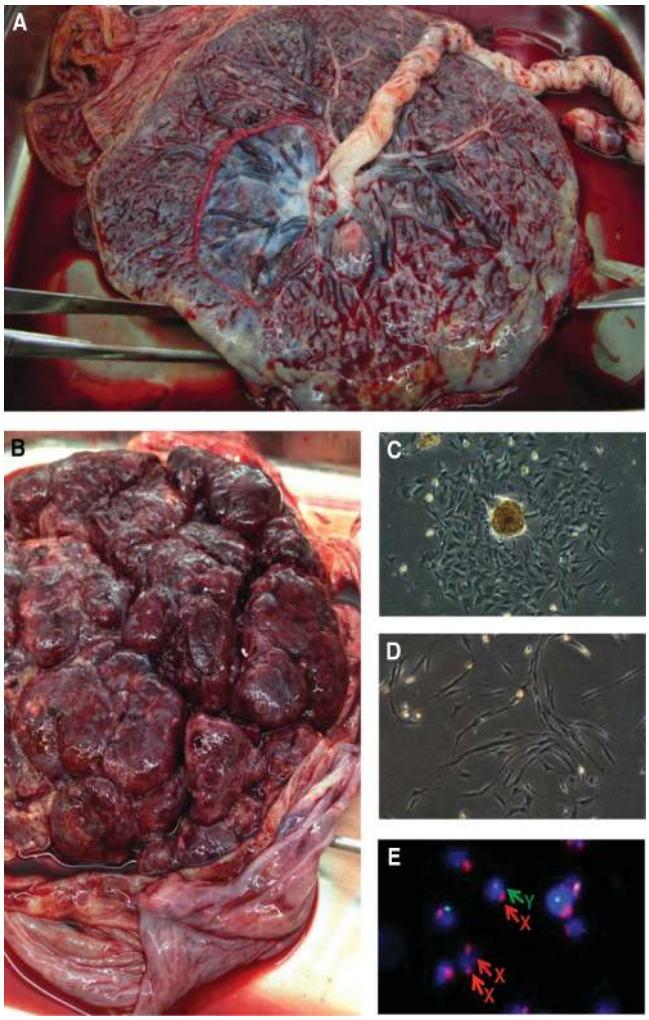



**Figure 5.1** The human term placenta and placental-derived MSCs. The fetal (A) and maternal (B) surfaces of the human term placenta. Note the umbilical cord on the fetal side and the cotyledons (lobes) on the maternal side. (C) Fetal CV-derived MSCs and (D) maternal MSCs 7 days after isolation using our novel method developed to ensure pure fetal MSCs (×10 magnification). (E) A representative image of XY chromosome fluorescence *in situ* hybridization (FISH) carried out on a sample of placental MSCs. Some cells have two red fluorescent signals, which indicate two X chromosomes; they are female and therefore maternal cells. Other cells have one green and one red fluorescent signal, which indicate one X and one Y chromosome; they are male and therefore fetal cells. (See color plate section.)

(around 50% cells versus 10%), whereas the AM-MSCs had greater adipogenic differentiation [41,49].

Samples obtained by different routes or methods have been determined to be selectively fetal or maternal in origin [44]. Amniotic fluid MSCs of fetal origin can be obtained by abdominal amniocentesis, whereas amniotic fluid sampling using the trans-cervical route obtained MSCs of mixed fetal and maternal origin [44].

In one study, the immunological effects of fetal MSCs mixed with maternal MSCs were greater than that of maternal MSCs alone [50]. Placenta-expanded stromal cells derived from pure maternal placental cell preparations increased the survival rate of irradiated mice from 27% in untreated animals to 68%. However, a mixture of maternal- and fetal-derived MSCs increased the cell survival rate by 98% [50]. This difference was attributed to the stronger inhibitory effect of fetal-derived MSCs on T cell proliferation at the feto-maternal interface compared with that of adult MSCs.

## 5.7 Consensus classification of human placental mesenchymal stem/stromal cells?

A classification of placental MSCs has been generated by the International Placenta Stem Cell Society (IPLASS) [51] and is shown in Table 5.1. The IPLASS recommendations for the characterization of human placental MSCs included the ISCT criteria for human BM-MSCs as well as niche-specific identification and characterization details. One specific criterion for placenta-derived MSCs was testing for fetal origin using methods sensitive enough to detect less than 1% maternal MSC contamination. In addition, as placenta-derived MSCs display a variable differentiation capacity, differentiation into only one or more mesodermal lineages (osteogenic, adipogenic, or chondrogenic) was suggested. These criteria were meant only to apply to amnion and chorion membrane-derived MSCs [41,47] as there are clear protocols for the isolation of fetal MSCs from these tissues. Parolini *et al.* also suggested that MSCs isolated from the CV are more likely to contain contaminating maternal MSCs and were thus excluded from this report [51].

## 5.8 Differentially isolating fetal or maternal mesenchymal stem/stromal cells from term placental villi

To date, one study has isolated and compared fetal and maternal MSCs from the same placenta. Patel *et al.* developed a method of $CD45^-$ $CD34^+$ magnetic and flow cytometric separation and culture to isolate fetal or maternal MSCs from term placenta [52]. Their primary goal was the isolation of placental EPCs from the CV [5]. Fetal MSCs were present in the $CD34^+$ $CD45^-$ fraction, while maternal MSCs were present in the $CD34^-$ $CD45^-$ fraction. In this study fetal MSCs required culture in endothelial growth medium, as was required for the placental EPCs [5,52].

## 5.9 Confounding factors for the isolation of fetal placental mesenchymal stem/stromal cells from chorionic villi

The IPLASS consensus report explicitly excluded maternal MSCs derived from placenta but did not give clear instruction

**Table 5.1** The defining characteristics of human BM-MSCs and placental stem cells.

| Source | ISCT [19] | IPLASS [51] |
|---|---|---|
| Definition | *Ex vivo* expanded human BM-MSCs for clinical use should conform to the following parameters:<br>**1.** Adherence to plastic<br>**2.** Fibroblast-like morphology<br>**3.** Differentiation *in vitro* to mesodermal lineages (adipocytes, osteoblasts, and chondrocytes)<br>**4.** >95% positive for mesenchymal cell surface markers CD105, CD73<br>**5.** <5% positive for mature hematopoietic and endothelial markers | **1.** Cells isolated from the placenta should be determined to be fetal in origin<br>**2.** Four regions of the placenta contain stem/progenitor cells: amniotic epithelial cells, AM-MSCs, chorionic mesenchymal stromal cells, and chorionic trophoblastic cells<br>**3.** Stem cell hierarchy and self-renewal may not be critical parameters of "stemness"<br>**4.** Placental membrane MSCs should conform to the ISCT criteria as described in column 1 of this table |
| Limitations | **1.** Not all of these characteristics correlate with MSCs from other organs or species<br>**2.** Does not include minimal criteria to address immune suppressive or anti-inflammatory properties of MSCs | **1.** Does not include maternally derived MSCs isolated by a number of groups from human placenta<br>**2.** Does not include MSCs from placental adnexa (e.g., umbilical cord, Wharton's jelly, and amniotic fluid)<br>**3.** Does not include placental stem/progenitor cells from other species |

on how to obtain pure fetal MSCs from the CV [51]. A systematic review also failed to determine why some groups isolated fetal and some maternal MSCs from the placenta [8]. No studies have addressed the mechanism responsible for maternal MSC contamination of *ex vivo* cultured term CV-derived MSCs, but this is likely due to the difficulty in avoiding maternal tissue even when the CV are dissected carefully using a dissecting microscope.

## 5.10 Assumptions from the literature: lack of data, specific assays, and specific methodological detail

It is sometimes assumed that MSCs isolated from the placenta will be fetal in origin since these cells grow faster than maternal cells [50]. Fukuchi *et al.* tested samples where the gender of the fetus was known and confirmed that three were male and three were female and assumed that the entire MSC population isolated was fetal in origin [12]. Jones *et al.* reported MSC-like chorionic stem cells to be fetal but provided no quantification of the fetal : maternal ratio despite utilizing both sex-determining region Y gene (*SRY*) polymerase chain reaction (PCR) and XY FISH testing [39]. Barlow *et al.* reported that in three of three informative cases in which the gender of the delivered baby was male, human placenta-derived MSCs were of maternal origin [17]. The lack of clear descriptions in the literature of the location of placental tissue isolated, different sample collection methods, and different enzymes used in the digestion of the placental tissue adds to the confusion and difficulty in the replication of results [39,41].

## 5.11 Methods for determining fetal and maternal mesenchymal stem/stromal cells in a cultured cell population

The origin of the placental MSCs has been examined by FISH [12,39,46,53–58], human leukocyte antigen (HLA) typing [44], karyotype analysis [17,46], or reverse transcription PCR for *SRY* or forensic markers [47,59,60]. The advantages and disadvantages of these methods are summarized in Table 5.2.

HLA typing and standard or quantitative PCR give an accurate representation of the dominant MSC donor in a population and can be used for placentas from both male and female babies, but they do not give information at the single cell level. Karyotype analysis is limited to only a few cells (between 6 and 20 cells in a population of millions), and is thus inadequate for high throughput characterization of a large population of *ex vivo* expanded cells.

XY FISH is the most common technique used to determine fetal : maternal cell ratio and was used in approximately 50% of studies investigating the origin of placenta-derived MSCs (reviewed in [8]). However, this method is only suitable if the placenta is from a male baby with one Y chromosome and one X chromosome, while MSCs of maternal origin will have two X chromosomes. MSCs derived from term decidua are always female and have been reported to proliferate faster than fetal MSCs derived from the amnion. Thus, even a small number of D-MSCs contaminating a fetal MSC source can outgrow the fetal MSCs in culture [44,46].

Detection of *SRY* (located on the human Y chromosome) by PCR has also been used as a method to determine the presence of maternal MSCs contaminating MSCs derived from a fetal source [59,61,62]. This technique can quickly and easily

**Table 5.2** Methods for detecting fetal MSCs and maternal MSC contamination.

| Method name | Result output | Advantages | Disadvantages |
|---|---|---|---|
| *SRY* PCR | PCR product: present = male absent = female | Determines the sex of the fetus | Determining maternal cell contamination in the placental setting is not possible |
| XY FISH | Immunofluorescence detection of red signal (X chromosome), and/or green signal (Y chromosome) | Male and female cells can be analyzed and represented as a percentage of the MSCs in the sample | Technically challenging, very expensive, time-consuming to manually count cells; however, could be automated. Can be used on fixed cells, frozen or paraffin embedded tissues |
| D1S80 PCR | 16 base pair standard tandem repeat (STR). Different band sizes that can represent different individuals | Detects the presence of various individuals in one sample confirmed by the respective controls. Can be used to detect contaminating maternal cells irrespective of the sex of the fetus. Quantitative analysis possible on digital PCR machines | Less effective because two individuals can have the same STR numbers and therefore needs to be used in combination with other techniques (e.g., full STR analysis) |
| STR PCR | Same as D1S80 but combines numerous STR sequences, including for sex chromosomes | Commercial quantitative STR analysis provides forensic quality results | Not economically feasible for a large number of experimental samples. Does not provide information on the origin of individual cells |

determine whether the sample is male. Additionally, it can be used on genomic DNA from archived samples such as fixed, frozen, or paraffin-embedded tissues.

## 5.12 A novel method to isolate fetal and maternal placental mesenchymal stem/stromal cells

In order to isolate CV MSCs we combined the dissection methods reported in the papers by Fukuchi *et al.*, Igura *et al.*, and Abumaree *et al.* [12,53,59]. This required starting from the maternal side of the placenta, removal of a whole cotyledon, followed by careful removal of the outside villous tissue to physically exclude maternal decidual cells. The 1 $cm^3$ of villous tissue from the center of the cotyledonary lobe was finely minced. We tested a variety of preparation conditions, including explant culture of CV tissue, enzymatic digestion protocols, and different culture media, including endothelial growth medium as described in [5]) in order to promote the *ex vivo* expansion and maximize purity of cultured fetal CV-MSCs. The only method that consistently supported the growth of the fetal CV-MSCs was dissection and collagenase digestion followed by explant culture in endothelial growth medium. Small pieces of tissue, similar to cell colonies or the "mushroom-like" colonies described by Nazarov *et al.* [54] remained in culture and were passaged with the cells over several passages.

We determined the gender of the cells obtained from each method using Y-chromosome-based PCR and FISH during the entire *ex vivo* culture period. We found that fetal CV-MSCs isolated by this method had superior growth and differentiation properties as well as different cell surface and genetic profiles that were not simply due to the gender difference between fetus and mother.

## 5.13 Understanding the maternal origin of the placental mesenchymal stem/stromal cells: the septa

In an intact gravid uterus the septa consist of anchoring columns between the cotyledon-composed extracellular matrix, fetal intermediate trophoblasts, and maternal decidual stromal cells [63]. Septa are formed by the folds in the decidua as a result of traction during villous development [63]. These placental septa can extend anatomically up to the chorionic plate [63]. Therefore, tissue samples taken from the fetal side and dissecting between cotyledons could contain maternal decidua cells. We found that taking CVsamples from the fetal side of the placenta, even as shallow as 1–5 mm under the chorionic plate, still resulted in collection of some decidual tissue and led to overgrowth by maternal MSCs.

## 5.14 Conclusions and future directions

We found that (1) fetal cells can be isolated and cultured from the term placenta free of maternal contamination, (2) dissection and culture methods are critical to exclude maternal cells, (3) serum plus the added growth supplements contained in EGM2 + 10 media are necessary for *ex vivo* proliferation of fetal CV-MSCs, and (4) standard MSC media such as Dulbecco's modified Eagle's medium and 10% fetal calf serum is advantageous for the *ex vivo* proliferation of maternal over fetal placental MSCs.

It is important in the future to determine the exact nutrients or growth factors required for fetal CV-MSC expansion and to provide detailed methods, including video or photographic images of the isolation procedure. Finally, thorough comparison of fetal and maternal MSCs from the same placenta has widespread implications for placental MSC banking and future regenerative medicine applications.

## Acknowledgments

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## References

1 M.A. Hill. Embryology. https://embryology.med.unsw.edu.au/embryology/index.php/Main_Page, 2014.
2 K. Moore, T. Persaud, M. Torchia. *The Developing Human: Clinically Oriented Embryology*. Elsevier/Saunders, 2013.
3 H. Abdulrazzak, D. Moschidou, G. Jones, P.V. Guillot. Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues. *J R Soc Interface* 2010;**7**(Suppl 6):S689–S706.
4 C. Gekas, K.E. Rhodes, H.K. Mikkola. Isolation and visualization of mouse placental hematopoietic stem cells. *Curr Protoc Stem Cell Biol* 2008; **Chapter 2**: Unit 2A.8.1-2A.8.14.
5 J. Patel, E. Seppanen, M.S. Chong, *et al.* Prospective surface marker-based isolation and expansion of fetal endothelial colony-forming cells from human term placenta. *Stem Cells Transl Med* 2013;**2**:839–847.
6 C. Pipino, P. Shangaris, E. Resca, *et al.* Placenta as a reservoir of stem cells: an underutilized resource? *Br Med Bull* 2013;**105**:43–68.
7 O. Parolini, M. Caruso. Review: preclinical studies on placenta-derived cells and amniotic membrane: an update. *Placenta* 2011; **32**(Suppl 2):S186–S195.
8 C.F. Heazlewood, H. Sherrell, J. Ryan, *et al.* High incidence of contaminating maternal cell overgrowth in human placental mesenchymal stem/stromal cell cultures: a systematic review. *Stem Cells Transl Med* 2014;**3**:1305–1311.
9 M. Crisan, S. Yap, L. Casteilla, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.
10 A.I. Caplan. Mesenchymal stem cells. *J Orthop Res* 1991;**9**:641–650.
11 M.F. Pittenger. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.
12 Y. Fukuchi, H. Nakajima, D. Sugiyama, *et al.* Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 2004;**22**:649–658.
13 P.V. Guillot, C. Gotherstrom, J. Chan, *et al.* Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 2007;**25**:646–654.
14 C.E. Gargett, H.P. Nguyen, L. Ye. Endometrial regeneration and endometrial stem/progenitor cells. *Rev Endocr Metab Disord* 2012;**13**:235–251.
15 C. De Bari, F. Dell'Accio, P. Tylzanowski, F.P. Luyten, Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001;**44**:1928–1942.
16 M.Q. Wickham, G.R. Erickson, J.M. Gimble, *et al.* Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clin Orthop Relat Res* 2003; (412):196–212.
17 S. Barlow, G. Brooke, K. Chatterjee, *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* 2008;**17**:1095–1107.
18 K. Bieback, S. Kern, A. Kocaömer, *et al.* Comparing mesenchymal stromal cells from different human tissues: bone marrow, adipose tissue and umbilical cord blood. *Biomed Mater Eng* 2008; **18**:S71–S76.
19 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.
20 R.A. Pelekanos, J. Li, M. Gongora, *et al.* Comprehensive transcriptome and immunophenotype analysis of renal and cardiac MSC-like populations supports strong congruence with bone marrow MSC despite maintenance of distinct identities. *Stem Cell Res* 2012;**8**:58–73.
21 L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**:2204–2213.
22 H. Wegmeyer, A. M. Broske, M. Leddin, *et al.* Mesenchymal stromal cell characteristics vary depending on their origin. *Stem Cells Dev* 2013;**22** (19):2606–2618.
23 D.G. Phinney, L. Sensebe. Mesenchymal stromal cells: misconceptions and evolving concepts. *Cytotherapy* 2013;**15**:140–145.
24 A. Keating. Mesenchymal stromal cells: new directions. *Cell Stem Cell* 2012;**10**:709–716.
25 L. Da Silva Meirelles, A.I. Caplan, N.B. Nardi. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 2008;**26**:2287–99.
26 P. Bianco, P.G. Robey, P.J. Simmons. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008; **2**:313–319.
27 S. Kern, H. Eichler, J. Stoeve, *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**:1294–1301.
28 B. Prunet-Marcassus, B. Cousin, D. Caton, *et al.* From heterogeneity to plasticity in adipose tissues: site-specific differences. *Exp Cell Res* 2006;**312**:727–736.
29 E. Ragni, T. Montemurro, E. Montelatici, *et al.* Differential microRNA signature of human mesenchymal stem cells from different sources reveals an "environmental-niche memory" for bone marrow stem cells. *Exp Cell Res* 2013;**319**:1562–1574.
30 C. Campagnoli, I. A. Roberts, S. Kumar, *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001;**98**:2396–2402.
31 P.V. Guillot, C. De Bari, F. Dell'Accio, *et al.* Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation* 2008;**76**:946–957.
32 Z.Y. Zhang, S.H. Teoh, M.S. Chong, *et al.* Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells* 2009;**27**:126–137.
33 Z.Y. Zhang, S.H. Teoh, M.S. Chong, *et al.* Neo-vascularization and bone formation mediated by fetal mesenchymal stem cell tissue-

engineered bone grafts in critical-size femoral defects. *Biomaterials* 2010;**31**:608–620.

34 Z.Y. Zhang, S.H. Teoh, J.H. Hui, *et al.* The potential of human fetal mesenchymal stem cells for off-the-shelf bone tissue engineering application. *Biomaterials* 2012;**33**:2656–2672.

35 P. De Coppi, G. Bartsch, Jr, M.M. Siddiqui, *et al.* Isolation of amniotic stem cell lines with potential for therapy. *Nat Biotechnol* 2007;**25**:100–106.

36 D. Moschidou, S. Mukherjee, M. P. Blundell, *et al.* Valproic acid confers functional pluripotency to human amniotic fluid stem cells in a transgene-free approach. *Mol Ther* 2012;**20**:1953–1967.

37 D. Moschidou, S. Mukherjee, M. P. Blundell, *et al.* Human mid-trimester amniotic fluid stem cells cultured under embryonic stem cell conditions with valproic acid acquire pluripotent characteristics. *Stem Cells Dev* 2013;**22**:444–458.

38 O.K. Lee, T.K. Kuo, W.M. Chen, *et al.* Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;**103**:1669–1675.

39 G.N. Jones, D. Moschidou, T.I. Puga-Iglesias, *et al.* Ontological differences in first compared to third trimester human fetal placental chorionic stem cells. *PLoS ONE* 2012;**7**:e43395.

40 A. Poloni, V. Rosini, E. Mondini, *et al.* Characterization and expansion of mesenchymal progenitor cells from first-trimester chorionic villi of human placenta. *Cytotherapy* 2008;**10**:690–697.

41 C.B. Portmann-Lanz, A. Schoeberlein, A. Huber, *et al.* Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. *Am J Obstet Gynecol* 2006;**194**:664–673.

42 P.I. Sipos, I.P. Crocker, C.A. Hubel, P.N. Baker. Endothelial progenitor cells: their potential in the placental vasculature and related complications. *Placenta* 2010;**31**:1–10.

43 E. Solder, B.C. Böckle, V.A. Nguyen, *et al.* Isolation and characterization of $CD133^+$ $CD34^+$ $VEGFR\text{-}2^+$ $CD45^-$ fetal endothelial cells from human term placenta. *Microvasc Res* 2012;**84**:65–73.

44 P.S. In 't Anker, S.A. Scherjon, C. Kleijburg-van der Keur, *et al.* Isolation of mesenchymal stem cells of fetal or maternal origin from human placenta. *Stem Cells* 2004;**22**:1338–1345.

45 S. Indumathi, R. Harikrishnan, R. Mishra, *et al.* Comparison of feto-maternal organ derived stem cells in facets of immunophenotype, proliferation and differentiation. *Tissue Cell* 2013;**45** (6):434–442.

46 P.A. Jaramillo-Ferrada, E.J. Wolvetang, J.J. Cooper-White. Differential mesengenic potential and expression of stem cell-fate modulators in mesenchymal stromal cells from human-term placenta and bone marrow. *J Cell Physiol* 2012;**227**:3234–3242.

47 M. Soncini, E. Vertua, L. Gibelli, *et al.* Isolation and characterization of mesenchymal cells from human fetal membranes. *J Tissue Eng Regen Med* 2007;**1**:296–305.

48 O.V. Semenov, S. Koestenbauer, M. Riegel, *et al.* Multipotent mesenchymal stem cells from human placenta: critical parameters for isolation and maintenance of stemness after isolation. *Am J Obstet Gynecol* 2010;**202**:193.e1–193.e13.

49 N. Sessarego, A. Parodi, M. Podesta, *et al.* Multipotent mesenchymal stromal cells from amniotic fluid: solid perspectives for clinical application. *Haematologica* 2008;**93**:339–346.

50 E. Gaberman, L. Pinzur, L. Levdansky, *et al.* Mitigation of lethal radiation syndrome in mice by intramuscular injection of 3D cultured adherent human placental stromal cells. *PLoS ONE* 2013;**8**:e66549.

51 O. Parolini, F. Alviano, G.P. Bagnara, *et al.* Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008;**26**:300–311.

52 J. Patel, A. Shafiee, W. Wang, *et al.* Novel isolation strategy to deliver pure fetal-origin and maternal-origin mesenchymal stem cell (MSC) populations from human term placenta. *Placenta* 2014;**35**:969–971.

53 K. Igura, X. Zhang, K. Takahashi, *et al.* Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy* 2004;**6**:543–553.

54 I. Nazarov, J.W. Lee, E. Soupene, *et al.* Multipotent stromal stem cells from human placenta demonstrate high therapeutic potential. *Stem Cells Transl Med* 2012;**1**:359–372.

55 M.I. Macias, J. Grande, A. Moreno, *et al.* Isolation and characterization of true mesenchymal stem cells derived from human term decidua capable of multilineage differentiation into all 3 embryonic layers. *Am J Obstet Gynecol* 2010;**203**:495.e9–495.e23.

56 N.M. Castrechini, P. Murthi, S. Qin, *et al.* Decidua parietalis-derived mesenchymal stromal cells reside in a vascular niche within the choriodecidua. *Reprod Sci* 2012;**19**:1302–1314.

57 H. Fazekasova, R. Lechler, K. Langford, G. Lombardi. Placenta-derived MSCs are partially immunogenic and less immunomodulatory than bone marrow-derived MSCs. *J Tissue Eng Regen Med* 2011;**5**:684–694.

58 G.G. Wulf, V. Viereck, B. Hemmerlein, *et al.* Mesengenic progenitor cells derived from human placenta. *Tissue Eng* 2004;**10**: 1136–1147.

59 M.H. Abumaree, M.A. Al Jumah, B. Kalionis, *et al.* Phenotypic and functional characterization of mesenchymal stem cells from chorionic villi of human term placenta. *Stem Cell Rev* 2013;**9**:16–31.

60 L. Wang, Y. Yang, Y. Zhu, *et al.* Characterization of placenta-derived mesenchymal stem cells cultured in autologous human cord blood serum. *Mol Med Rep* 2012;**6**:760–766.

61 J.C. Polanco, P. Koopman. *Sry* and the hesitant beginnings of male development. *Dev Biol* 2007;**302**:13–24.

62 P. N. Goodfellow, R. Lovell-Badge. *SRY* and sex determination in mammals. *Annu Rev Genet* 1993;**27**:71–92.

63 K. Benirschke, G.J. Burton, R.N. Baergen, *Pathology of the Human Placenta*. Springer, 2012.

SECTION III

# The cellular and molecular biology of mesenchymal stromal cells

CHAPTER 6

# Epigenetic regulation of mesenchymal stem/stromal cell growth and multipotentiality

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## 6.1 Introduction

Mesenchymal stem/stromal cells (MSCs) are considered to be a reliable source of stem cells for cellular-based regenerative medicine applications owing to their multipotent potential, proangiogenic, and immunomodulatory properties. While the use of MSCs is less controversial than embryonic-derived stem cells (ESCs), they, like all somatic stem cells, have a finite lifespan *ex vivo*. Key master regulatory genes and signaling pathways critical for MSC self-renewal and lineage determination are the subjects of intense investigations. However, the role of epigenetic modifiers in regulating the chromatin structure and accessibility of DNA on genes of various signaling pathways has only recently been investigated. With the increasing evidence implicating epigenetic modifiers in MSC differentiation, growth, and the correlation between mutations in epigenetic modifiers resulting in human disorders with musculoskeletal phenotypes, these findings suggest the importance of these proteins in skeletal tissue development.

Stem cells are characterized by their capability to self-renew and to terminally differentiate into multiple mature cell types. Somatic or adult stem cells have a finite self-renewal capacity and are lineage restricted. The use of adult stem cells for therapeutic purposes has been a topic of enormous interest given the ethical considerations associated with ESCs. Mesenchymal stem cells are adult stem cells that can differentiate into osteogenic, adipogenic, chondrogenic, or myogenic lineages. Owing to their ease of isolation and their unique characteristics, including lack of immunogenicity in their recipient, MSCs have been widely regarded as potential candidates for tissue engineering and repair. While various signaling molecules important to MSC differentiation have been identified, our complete understanding of this complex process is lacking.

Recent investigations focused on the role of epigenetic regulation in lineage-specific differentiation of MSCs have shown that unique patterns of DNA methylation and histone modifications play an important role in the induction of MSC differentiation toward specific lineages. Nevertheless, MSC epigenetic profiles reflect a more restricted differentiation potential compared with ESCs. Here, we review the effect of epigenetic modifications on MSC multipotency and differentiation, with a focus on osteogenic and adipogenic differentiation. We also highlight clinical applications of MSC epigenetics and nuclear reprogramming.

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## 6.2 Mesenchymal stromal/stem cells

Bone marrow MSCs (BM-MSCs) are tissue-specific postnatal stem cells that are restricted in their differentiation potentials to form functional skeletal tissues and myelosupportive stroma. Mesenchymal stem-like cells have also been identified in other tissues, such as peripheral adipose tissue, umbilical cord blood, amniotic fluid, placenta, dental pulp, periodontal ligament, tendons, synovial membrane, and skeletal muscle [1–13]. However, these different MSC-like populations exhibit wide variations in their multidifferentiation potentials, hematopoietic supportive properties, and proliferation capacities. This variation is most likely due to the different developmental origins and tissue-specific nature of the different MSC-like populations, which may be under the direct control of epigenetic patterning.

BM-MSCs were first identified as clonogenic stromal precursor cells, termed colony-forming unit–fibroblastic (CFU-F) within bone marrow aspirates of rodents [14] and subsequently in other species, including humans [15]. A common feature of the *ex vivo* expanded progeny of CFU-Fs is their heterogeneity with respect to morphology, ability to undergo self-renewal, myelosupportive capacity, lifespan, and multidifferentiation potential [1,16–19]. These observations have led to the proposal that the stromal component within bone marrow is comprised of a hierarchy [20] of immature stem cells, committed progenitor cells, and mature functional stromal populations (Figure 6.1)

Since the vast majority of CFU-Fs demonstrate limited growth and differentiation potential, the general term describing MSCs should only refer to the *ex vivo* expanded progeny of a minor subset of long-lived multipotential CFU-Fs, with the potential to form osteoblasts, adipocytes, chondrocytes, smooth muscle cells, and myelosupportive fibroblasts *in vitro* or when transplanted *in vivo*. While attempts have been made to establish an immunophenotype specific for MSC-like populations (HLA-ABC$^+$, CD73$^+$, CD105$^+$, CD29$^+$, CD13$^+$, CD44$^+$, CD90$^+$, CD45$^-$, CD34$^-$, CD31$^-$, and CD14$^-$ [21,22], the cell surface markers used are generic to a wide range of cultured fibroblastic cells and some vascular and hematopoietic cell populations with different growth and developmental potentials. Moreover, none of the positive selection markers (HLA-ABC$^+$, CD73$^+$, CD105$^+$, CD29$^+$, CD13$^+$, CD44$^+$, and CD90$^+$) are capable of distinguishing between immature and committed stromal cell lineages or non-MSC fibroblasts. The identification of multipotential clonogenic MSCs from aspirates of human bone marrow has been achieved based on their high expression of the stromal precursor cell surface antigen STRO-1, which is downregulated in committed stromal lineages. Purification of STRO-1$^{bright}$ BM-MSCs was achieved by dual fluorescence-activated cell sorting based on their co-expression of the perivascular markers CD106 and CD146 and their lack of expression of the hematopoietic markers CD34, CD14, CD45, and glycophorin-A [4,16,23,24]. Therefore, the development of immunoselection protocols to obtain purified populations of BM-MSCs has allowed the identification of putative regulatory genes critical for maintaining the MSC population following *ex vivo* expansion [25–27].

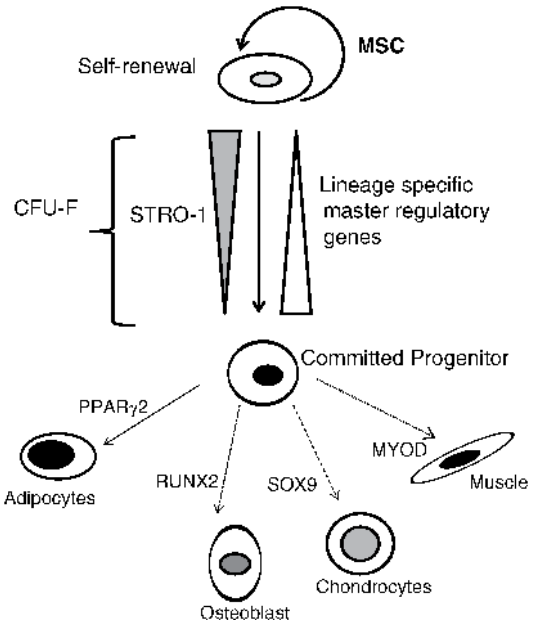



**Figure 6.1** Stromal hierarchy of MSCs. BM-MSCs contain a heterogeneous population of stem cell progenitors and committed lineage progenitors. Stem cell progenitors (MSCs) express high levels of STRO-1 and low levels of lineage-specific master regulatory genes. These cells have the capacity to self-renew and differentiate. Committed lineage progenitors express low levels of STRO-1 and higher expression of specific master regulatory genes. As the committed progenitors differentiate they express key master regulator genes such as *PPAR-γ2*, *RUNX2*, *SOX9*, and *MYOD* as MSCs differentiate into mature adipocytes, osteoblasts, chondrocytes, and muscle cells respectively.

Understanding the processes that drive BM-MSC lineage commitment may one day allow us to manipulate MSCs to differentiate down specific lineages required for cellular therapies where stromal cells are defective in their differentiation and renewal capacity. Signaling pathways such as Wnt, Notch, bone morphogenetic protein (BMP) [28] and key transcription factors RUNX2, PPAR-γ2, MOYD, and SOX9 have been implicated as critical drivers of MSC lineage commitment. The activation of these signaling pathways and expression of transcription factors are dependent on the regulation of chromatin, allowing the activation of these genes and their targets. Therefore, it is important to identify which epigenetic modifications are critical for the regulation of MSC growth and lineage specification. This chapter will identify the relationship of important epigenetic modifications involved in MSC maintenance, self-renewal, and differentiation.

## 6.3 Epigenetics

Epigenetics is the cellular modification of reversible and heritable changes in gene expression that occur without changes in the

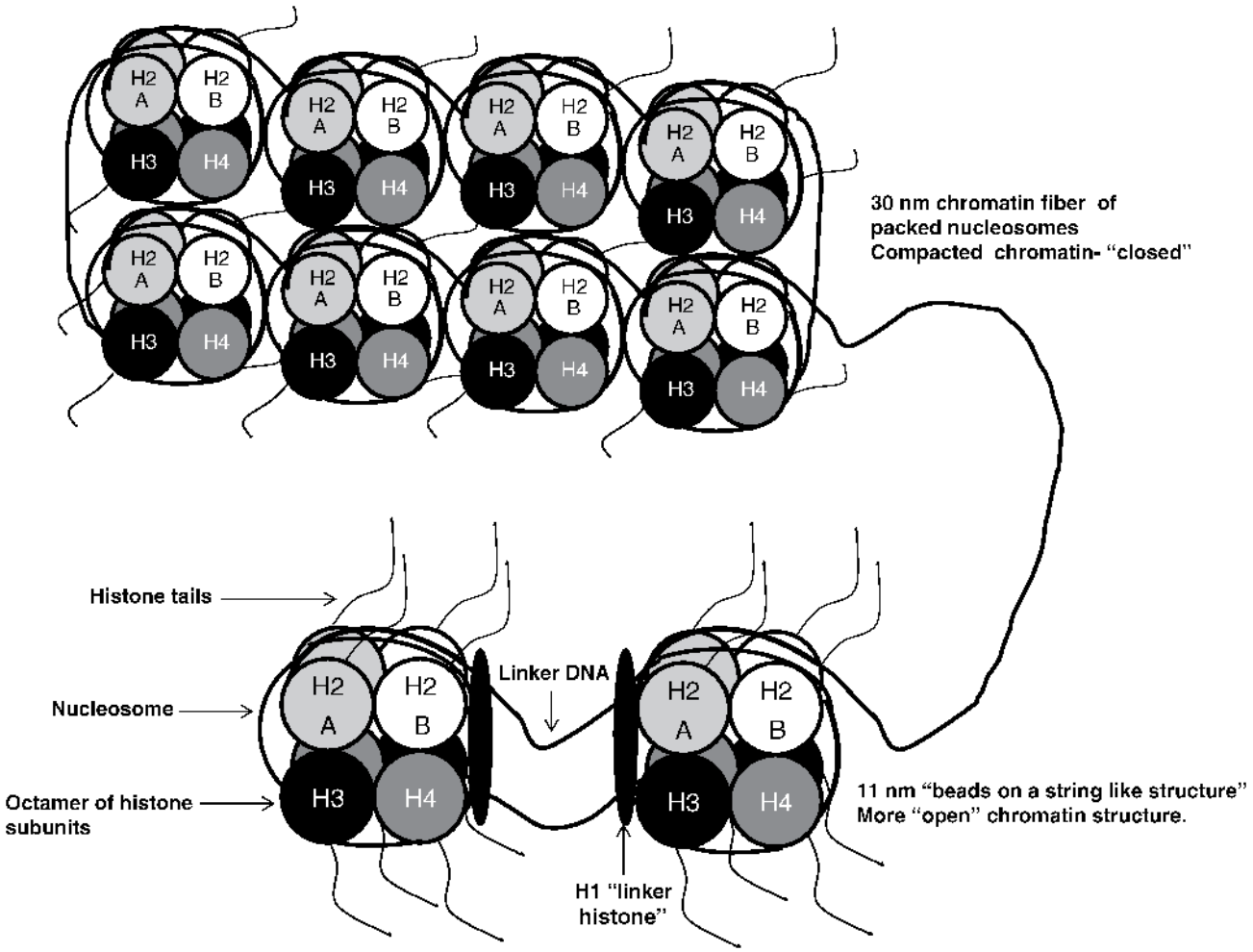



**Figure 6.2** Compaction of DNA through the formation of chromatin. DNA is compacted within a cell through chromatin. 145–146bp DNA is wrapped around a nucleosome structure consisting of an octamer of histone subunits, two copies of H2 A, H2 B, H, and H4. H1 "linker histone" anchors the DNA wrapped around the nucleosome in place. A region of DNA is present in between nucleosomes known as linker DNA. Histone tails protrude out of the histone subunits and these are regions of posttranslational modifications that dictate a more open ("beads on a string") or closed chromatin structure. Nucleosomes are further compacted to form a 30 nm closed chromatin fiber structure. The structure can be further condensed to chromosomes.

DNA code [29]. Epigenetic modifications such as DNA methylation and posttranslational modifications include histone modifications and chromatin remodeling. These molecules regulate the structure of chromatin, determining the accessibility of genes to transcription factors and other modulators involved in gene regulation. Chromatin is formed through the compaction of a large amount of DNA where 145–146 base pairs of DNA are wrapped around a nucleosome, consisting of an octamer of histone subunit [30,31] (Figure 6.2).

Nucleosomes are joined together by linker histone 1 (H1) and a small region of DNA, which form a "beads-on-a-string-like structure." Each histone subunit has a specific sequence of amino acids in a tail-like structure defined as a "histone tail" that protrudes out from the nucleosomes and can be posttranslationally modified (Figure 6.2).

The functional state of chromatin can be modified through C5 methylation of DNA at cytosines within cytosine–phosphate–guanosine (CpG) dinucleotides and the posttranslational modification of histone proteins and residues within the histone tails (Figure 6.2). DNA methylation is commonly located at the cytosine of the dinucleotide sequence CpG and is often located at genes that have tissue-restricted expression and become stably silent. The activity of DNA methyltransferases (DNMTs) is critical for genome stability, X inactivation, genomic imprinting, and regulating key developmental genes during cellular differentiation [32–42]. DNA methylation can be separated into two types: *de novo* and maintenance. In early mammalian development the mature oocyte, primordial germ cells, and blastula DNA are relatively unmethylated. During embryogenesis and the formation of the three germ layers, DNA methylation plays a pivotal role in patterning tissue-specific expression [43–45].

DNMT1, known as the "maintenance methyltransferase," is responsible for copying methylation patterns after DNA replication, imprinting, X activation, and CpG methylation [38,46].

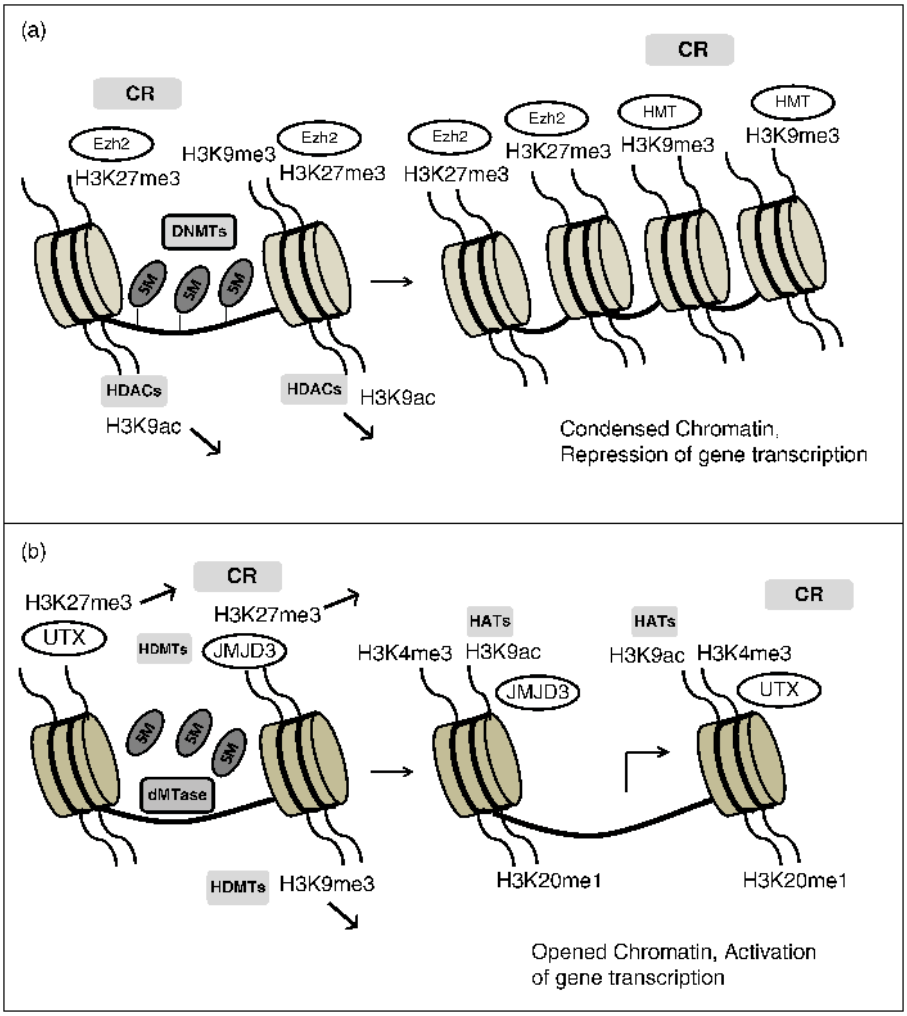



**Figure 6.3** Chromatin-modifying proteins modulate chromatin structure. DNA accessibility is dictated by chromatin structure; chromatin can be compacted or opened up, leading to repression or activation of genes through DNA and posttranslational histone modifications (PTMs). (a) 5-Methyl cytosine methylation (5M) of DNA by DNMTs. Histone 3 lysine 27 methylation by HMTs can trimethylate histone 3 lysine 9 and 27 (H3K27me3 (Ezh2)) and the removal of H3K9 acetylation (ac) by HDACs. All these epigenetic modifications facilitate the compaction of the chromatin structure and gene repression. (b) Removal of 5M from DNA by DNA demethylases (dMTase). The removal of H3K9me3 and H3K27me3 is mediated by UTX and JMJD3 histone demethylases (HDMs). The addition of H3K4me3 and H3K420me1 methylation by HMTs and H3K9ac by HATs lead to the opening up of chromatin and activation of gene transcription. Chromatin remodelers (CRs) are recruited to chromatin by a combination of DNA and PTM leading to either the unravelling or condensation of chromatin, depending on the histone code cellular signals.

Gene silencing can occur via DNA methylation of CpG islands to prevent the binding of proteins to DNA. This in turn acts as a marker for CpG-binding domain proteins (MBD), which recruit histone deacetylases (HDACs) and histone 3 lysine 9 (H3K9) methyltransferase, allowing the compaction of chromatin [47–52]. DNA methylation can then be modified by DNA demethylases, which remove the cytosine di-methylation modification allowing the recruitment of factors involved in transcriptional activation. The 10-11-translocation 1–3 (TET1–3) proteins are dioxygenases that form 5-hydroxymethylation (5hmc), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC), which are intermediates that form in the process of the removal of methylation [53,54]. With the development of methyl-DNA immunoprecipitation microarray hybridization and bisulfite sequencing [55,56], investigations examining the role and patterns of DNA methylation in different processes such as development, stem cell self-renewal, and pluripotency have yielded an enormous amount of information showing methylation of developmental genes as a cell becomes more restricted in its multipotent capacity and less stem cell-like [57,58]. Posttranslational modification of histones is another level of transcriptional regulation that can result in repression or activation of gene expression. The structure of chromatin can be regulated through methylation, acetylation, phosphorylation, sumoylation, and ubiquination of histone tails. Posttranslational modification of 28 known residues gives rise to over 100 million differentially modified nucleosomes within cells, accounting for the great diversity of signals and gene expression outcomes [59]. The regulation of DNA through epigenetic modifications allows the specification and maintenance of cellular identity in a wide range of cell types [37,60]. Acetylation of histones by histone acetyl-transferases (HATs) leads to the activation of transcription, whereas, histone methylatation by histone methyltransferases (HMTs) can regulate gene activation or repression depending on which residues are methylated. Histones can be mono- (me1), di-, (me2) or trimethylated (me3) on lysine

residues and me1 or me2 on arginine residues. Methylation of histone 3 lysine 4 (H3K4), lysine 36 (H3K36), and lysine 79 (H3K79) is associated with active transcription, and methylation of H3K9 and lysine 27 (H3K27) is associated with gene repression [61] (Figure 6.3).

With the development of chromatin immunoprecipitation (ChIP) techniques researchers can now identify histone proteins that are associated with a particular region of the genome. Recent investigations of ESCs using microarray and ChIP techniques have revealed the presence of both H3K27me3 and H3K4me3 specific modification patterns on chromatin termed "bivalent domains." The presence of both these bivalent domains within ESC genomes allows the silencing of developmental regulators whilst keeping them poised for activation. Upon ESC differentiation these bivalent domains become univalent, allowing the activation of genes that drive lineage commitment [62]. Methyltransferases and demethylases are thought to be responsible for the switch between repressive H3K27 domains and active H3K4 domains. Different combinations of histone modifications can influence the recruitment of different effector proteins and transcription factors to chromatin, and these combinations are known as the "histone code," which determines the functional state of chromatin [63,64].

Furthermore, the interplay between the different epigenetic modifying enzymes (HATs, HDACs, DNMTs, MBD, and HMTs) is critical for normal gene transcription, suggesting the importance of epigenetic gene regulation in many cellular processes.

## 6.4 DNA methylation and histone modifications in mesenchymal stem/stromal cells

The importance of key transcription factors and signaling pathways in regulating MSC differentiation, growth, and lifespan has been widely investigated. However, the role of epigenetic modifiers in regulating chromatin structure allowing the activation or repression of these key genes and signaling pathways in MSCs has only recently been explored [65].

Studies have assessed the DNA methylation profiles between different MSC-like cells derived from bone marrow, adipose tissue, and skeletal muscle using bisulfite sequencing, methyl-DNA immunoprecipitation, and promoter array hybridization (MeDIP-chip) [66]. Analysis of reference sequence promoter arrays (Re-Seq) revealed that promoter DNA methylation patterns were similar in MSC-like populations derived from adipose tissue, bone marrow, or skeletal muscle [67]. It was proposed that a core of hypermethylated genes constituted a common intrinsic epigenetic marking of different MSC-like populations, lending support to the common features of MSC-like cells identified in various tissues. It is suggested that promoter methylation patterns may dictate gene expression, where strong methylation dictates repression, while hypomethylation in some tissues determines either repressive or active gene expression when complemented with other modifying proteins. Furthermore, promoter DNA methylation is only one part of the regulation of lineage determination since low levels of DNA methylation are not a reliable predictor of differentiation capacity [66,68,69]. These studies further suggested that the overall CpG methylation status of genes is not indicative of differentiation potential [70–72], implying that processes other than DNA methylation contribute to the differentiation capacity and lifespan of MSCs. Therefore, the role of other posttranslational histone modifiers is becoming increasingly more important in the study of stem cell biology. With the use of techniques such as chromatin immune-precipitation sequencing (ChIP-Seq) it is now possible to identify the epigenetic patterns of cells during many cellular process under varying environmental conditions.

## 6.5 Epigenetic regulation of osteogenic differentiation

Osteogenic differentiation of MSCs is controlled by key signaling pathways and transcription factors that can be regulated through epigenetic modifications [73]. For example, DNA methylation and demethylation have been reported to play a role during osteogenic differentiation where highly methylated osteogenesis-associated genes *DLX5*, *RUNX2*, *BGLAP*, and *OSTERIX* in adipose-derived MSCs undergo demethylation and increased gene expression during osteogenic differentiation [7]. Similarly, alkaline phosphatase, *Trip10*, and rank ligand have also been shown to be regulated by DNA methylation during BM-MSC osteogenic differentiation [75–77], while another report observed reduced gene expression levels of the stem-cell-associated genes *BRACHYURY* and *LIN28* by hypomethylation of the promoter during BM-MSC differentiation [78]. Furthermore, the intrinsic and extrinsic signals that MSCs receive during maturation can also govern the epigenetic regulatory outcome of MSC cell fate determination [79].

Besides DNA methylation, gene repression can be mediated through H3K27 methylation. Studies have found that the H3K27 methyltransferase Enhancer of Zeste Homolog 2 (*EZH2*), a component of the polycomb repressor complex 2 (*PCR2*), can interact with DNMTs to promote gene repression [80]. EZH2 has been widely implicated as a regulator of developmental processes and differentiation-associated genes [81,82]. EZH2 activity can lead to either mono-, di-, or trimethylation of the K27 residue of H3 tails (H3K27me1/2/3) and is associated with heterochromatin, chromatin compaction, and repression of gene transcription [83–85]. EZH2 influences chromatin structure through adding an me3 modification to H3K27, where this mark is recognized by PRC1, which recruits effector proteins such as chromatin remodelers involved in compaction of chromatin [86]. EZH2 deficiencies in mice lead to perinatal mortality due to deformities in morphological movement of ESCs in the developing embryo.

More recently it was shown that overexpression of *EZH2* in human BM-MSCs inhibited osteogenic differentiation, while siRNA knockdown of *EZH2* or treatment with the methyltransferase inhibitor DZNep promoted osteogenic differentiation, identifying a direct role for EZH2 in regulating bone cell differentiation of human BM-MSCs [87]. Furthermore, EZH2 was found to bind and methylate the promoter regions of RUNX2 and osteopontin using ChIP analysis [87], suggesting that EZH2 may be a key regulator for MSC cell fate determination. During MSC differentiation, cyclin-dependent kinase 1 (CDK1) represses EZH2 methyltransferase activity through the phosphorylation of residue threonine 487, thus promoting BM-MSC osteogenic differentiation *in vitro* [88]. ChIP-on-chip analysis of adipocytes and osteoblasts revealed that the myocyte enhancer factor-2 interacting transcription factor (MITR or HDAC9c) is bound by EZH2 in adipocytes but not in osteoblasts. During osteogenesis, EZH2 binding to MITR is repressed, allowing MITR to complex with PPAR-γ2, thereby directly interrupting PPAR-γ2 activity [89]. These observations have led to the hypothesis that the known H3K27 demethylases (HDMs) KDM6A (UTX – ubiquitously transcribed tetra-tricopeptide repeat X) and Jumonji domain-containing protein 3 (JMJD3, also known as KDM6B) could also be pivotal in dictating the lineage specificity of MSCs.

Histone methylases and demethylases control the level of methylation present within a cell. An increase in H3K27 demethylation leads to the recruitment of gene-activating factors and activation of gene transcription. UTX and JMJD3 have both been identified as having potential roles in regulating MSC osteogenic differentiation [87,90–93]. *UTX* overexpression in MSCs was found to promote osteogenic differentiation, while siRNA knockdown of *UTX* resulted in decreased osteogenesis *in vitro* and *in vivo* [87]. However, the role of JMJD3 in osteogenic differentiation is thought to act by regulating RUNX2 and OSTERIX expression in osteoblasts [91–93], which is regulated by MIR146A during osteogenic differentiation [92]. Therefore, it is still unclear whether UTX and JMJD3 work synergistically or individually to regulate osteogenic differentiation. While it is well established that the BMP, Wnt, and Notch signaling pathways all play important roles in osteoblast differentiation [28], these two demethylases could play different roles in regulating osteogenic differentiation based on the cellular signals they encounter leading to specialized effects on cellular differentiation.

Methylation of another lysine residue on H3, H3K9, is associated with chromatin compaction and gene repression. H3K9me and H3K27me modifications are usually present on silenced genes, where H3K9 methylation can depend on the H3K4 methylation status of the adjacent lysine residue [94]. There are also multiple known H3K9 HMTs, including SUV39h, Set domain bifurcated 1 (SETDB1), G9a, and Set7/Setd8, which are potentially involved in the global enrichment of H3K9Me2 during MSC osteogenic differentiation [95]. Other studies have demonstrated that the activation of the noncanonical Wnt signaling pathway resulted in promotion of osteogenic differentiation due to H3K9 methylation and repression of PPAR-γ2 [96]. During osteogenic differentiation the promoter regions of *ALP*, *RUNX2* and *OP* were acetylated and underwent H2K9me2 demethylation. Therefore, it appears that the addition of acetylation and removal of H3K9me3 facilitates the expression of bone-associated genes to help drive osteogenesis. Conversely, stem-cell-associated genes such as *OCT4* and *NANOG* were downregulated with a corresponding deacetylation and increase in H3K9me3 on the promoters of these genes. Furthermore, KDM4B, a H3K9 demethylase, was found to remove H3K9me3 and H3K27me3 from osteogenesis-associated genes such as *HOX* and *DLX*, resulting in the opening up of chromatin and promotion of MSC osteogenic differentiation [90].

H3K4 methylation has been shown to positively regulate transcription by recruiting nucleosome remodeling enzymes and histone acetylases [97]. H3K4 methylation is mediated by SET domain-containing methyltransferases, such as mixed lineage leukemia 1–5 (MLL1–5), SET1A/B, and SET7/9. H3K4 is critical for gene activation, where this histone modification mark is widely expressed on genes involved in differentiation, growth, and self-renewal [98]. The balance of H3K4 methylation is controlled by H3K4 HMTs and H3K4 HDMs. MLL proteins often associate in a complex with HDM UTX/KDM6A or JMJD3/KDM6B and Pax transactivation domain-interacting protein (PTIP; nuclear protein associated with active chromatin), allowing the rapid removal of the repressive methylation and the application of the active methylation H3K4 [99].

Opening up of chromatin to allow for gene transcription is regulated by active histone modifications such as H3K4me3. Studies have identified that H3K4me3 and hyperacetylation mediated by HOXA10 induces chromatin remodeling, facilitating *RUNX2* activation and its downstream target gene, *OSTEOCALCIN* [100]. H3K4 HDMs are associated with removal of the H3K4 active methylation modification involved in transcriptional activation. Jumonji domain-containing histone demethylases such as the JARID1 family of HDMs (JARID1A–D) remove H3K4me3 and H3K4me2 modifications. JARID1A inhibits osteogenesis through the promoter demethylation of *RUNX2*, *OSTERIX*, *OSTEOCALCIN*, and *ALKALINE PHOSPHATASE* in human adipose-derived MSCs [101]. The Nucleolar protein 66 is also known to have H3K4me3/2/1 demethylase activity and has been found to demethylate the promoter region of *OSTERIX* and interact directly with *OSTERIX* preventing activation of its downstream target genes [102]. Furthermore, in human adipose-derived MSCs, osteogenic differentiation was found to be regulated by the H3K4 demethylase retinoblastoma-binding protein 2 (RBP2). Knockdown of *RBP2* increased H3K4me3 and promoted *ALKALINE PHOSPHATASE*, *OSTEOCALCIN*, and *OSTERIX* expression. Furthermore, RBP2 directly associates with the RUNX2 protein, where knockdown of *RPB2* leads to activation of *RUNX2* [101].

Other modifications, such as acetylation of the histone tails lead, to neutralization of the partial electric charge of lysine, which in turn results in opening of the chromatin structure.

HATs and HDACs are responsible for the maintenance of cellular histone acetylation. H3K9 and H4K16 acetylation (H3K9ac and H4K16ac) are commonly associated with euchromatin and transcriptional activation. During osteogenic differentiation, H3K9ac enrichment decreased on the stem-cell-associated genes *NANOG* and *OCT4* and were enriched with an increase in H3K9me2 [95].

Following long-term *ex vivo* expansion, MSCs undergo spontaneous osteogenic differentiation with an increase in *RUNX*2 and *ALKALINE PHOSPHATASE* expression. This observation has been correlated with an increase of H3K9ac and H3K14ac modifications on these osteogenic genes [103]. There are 18 known HDACs that contribute to skeletal development and bone homeostasis. Many of these HDACs regulate bone formation and development through the regulation of *RUNX*2 [104–107]. H3 and H4 hyperacetylation was identified on osterix and osteocalcin promoters, leading to decreased HDAC1 recruitment to these promoters [108]. Furthermore, the treatment of MSCs with the HDAC inhibitors vorinostat, sodium butyrate, valproic acid, and trichostatin A was found to promote osteogenic differentiation [109–114].

These studies have identified a wide range of DNA and histone posttranslational modifications directly involved in the osteogenic differentiation of MSCs, but have yet to determine the sequential and temporal activities of these epigenetic modifiers during the process of osteogenesis.

## 6.6 Epigenetic regulation of adipogenic differentiation

Adipogenic differentiation of MSCs is thought to be regulated, in part, by DNA methylation, where CpG sites have been shown to be hypomethylated in key adipogenic genes such as *PPAR-γ2*, *C/EBP-α*, *leptin*, *fatty acid-binding protein 4* and *lipoprotein lipase* in *ex vivo* expanded adipose-derived MSCs [70].

The H3K27 methyltransferase EZH2 is also known to promote adipogenesis by repressing Wnt-1, -6, -10a, and -10b, leading to disruption of Wnt/β-catenin signaling rather than directly regulating *PPAR-γ2* [115]. In human studies, overexpression of *EZH2* in BM-MSCs promoted adipogenic differentiation, while siRNA knockdown inhibited adipogenic differentiation [87]. Overexpression of the HDM UTX inhibited adipogenesis, while siRNA knockdown promoted adipogenic differentiation of BM-MSCs [87], possibly by demethylating Wnt-associated genes, therefore inhibiting adipogenesis indirectly. Other studies have shown that knockdown of H3K27me3 demethylase JMJD3/KDM6B and H3K4me3 demethylase KDM4B inhibit adipogenesis, further supporting the role of demethylases in adipogenic differentiation [90]. Additional research has shown that, during adipogenic differentiation, histones bound to adipogenesis-associated gene promoters undergo H4K20me1 methylation mediated by the methylase, PR-Set7/Setd8 [116]. During MSC adipogenic differentiation the H3K9 HMT Suv39h1 mediates activating enhancer binding protein 2-α (AP-2-α)-dependent inhibition of CCAAT-enhancer-binding protein (C/EBP-α) [117]. Furthermore, *PPAR-γ2* target gene expression is blocked by SUV39H1, suggesting that H3K9 methylation is a negative regulator of adipogenesis [118]. Similarly, Wnt5a-induced H3K9 SETDB1 methylation was found to activate the noncanonical Wnt signaling cascade, resulting in the inhibition of *PPAR-γ2* function and adipogenesis, while promoting osteogenic lineage commitment [119,120]. In another report the HMT G9a was found to be responsible for di-methylation of H3K9, which regulates gene repression [121]. Furthermore, ChIP-Seq analysis of preadipocytes revealed that H3K9me2 and H3K27me3 modifications at gene loci were largely nonoverlapping [122]. In these studies, *WNT* gene loci exhibited high levels of H3K27me3 and low levels of H3K9me2, while *PPAR-γ2* was found to have high levels of H3K9me2 and low levels of H3K27me3. Inhibition of G9a methylation activity was also found to increase *PPAR-γ2* gene expression, leading to promotion of adipogenesis. While G9a represses *PPAR-γ2* expression in an HMT activity-dependent manner, it has also been shown to facilitate *WNT10A* expression independent of its enzymatic activity [122]. Therefore, WNT10A facilitates β-catenin stabilization, leading to the inhibition of adipogenesis and promotion of osteogenic commitment.

Lysine-specific demethylase 1 (LSD1), also known as KDM1, forms a complex with androgen receptors and can act as an H3K9 di-demethylase. LSD1 can regulate C/EBP-α by decreasing H3K9me2 and maintaining H3K4me2 at its promoter, counteracting the activity of the H3K9 methyltransferase, SETDB1 [123]. Activating methylation modifications such as H3K4 play a critical role in activation of key adipogenic differentiation genes. MLL3 and MLL4 are mono- and di-methyltransferases targeting H3K4, which are required for enhancer activation, gene activation, and differentiation and are critical for *PPAR-γ* and *C/EBP-α* expression during adipogenesis [99,124–126]. Moreover, H3K4 di-methylation, a mark of poised genes, has been shown to be present on the promoters of the adipose-associated genes *APM1*, *GLUT4*, *GPD1*, and *LEPTIN* in preadipocytes [98,127].

Other studies have shown that H4K20 methylation, often associated with gene repression, can aid in gene activation via mono-methylation. H4K20 mono-methyltransferase *PR-SET7/SETD8* gene is upregulated by PPAR-γ2 during adipogenesis, and knockdown of *PR-SET7/SETD8* suppressed adipogenesis [116]. These findings suggest that combinations of lysine methylation patterns are required for promotion or repression of adipogenesis.

The histone 3 argine 8 methylase (H3R8) known as the protein arginine methyltransferase (PRMT) family of enzymes is divided into two classes. Type I PRMTs asymmetrically mono- and di-methylate arginine residues, whereas type II PRMTs symmetrically mono- and di-methylate arginine residues [128]. Both types of PRMT form complexes with SWItch/Sucrose Non-Fermentable (SWI/SNF) ATP-dependent chromatin-remodeling enzymes, adding another functional layer on to chromatin

regulation. Knockdown of *PRMT5* inhibits adipogenic differentiation and adipogenic gene expression, while overexpression accelerates adipogenic differentiation [129]. The presence of PRMT5 recruits SWI/SNF binding of *PPAR-γ2* and downstream adipose-associated target genes allowing the opening up of chromatin and activation of gene transcription. The Brg1 SWI/SNF remodeling enzyme appears to play a critical role in remodeling chromatin at the PPAR-γ2 gene locus to facilitate *PPAR-γ2* gene expression [130].

Histone acetylation has been reported to be an important modification in regulating white adipocyte differentiation. HATs promote the recruitment of factors involved in opening up of chromatin and the activation of key genes critical for adipogenesis [131,132]. HATs such as H3K27ac CBP/p300 have been shown to be involved in mediating *PPAR-γ2* gene expression and promoting adipogenesis in 3T3-L1 preadipocytes. Furthermore, genome-wide profiling of H3K9ac and H3K27ac in 3R3-L1 preadipocytes identified their presence on the *PPAR-γ2* gene locus during adipogenesis. However, little is known of the role of CBP/p300 and other HATs in the regulation of *PPAR-γ2* and other key adipogenic genes in human MSCs.

The removal of acetylated histones is important for gene repression during maturation where adipogenic differentiation can be repressed through the unphosphorylated form of retinoblastoma (Rb) protein, which recruits HDAC3 to the promoter of *PPAR-γ2* preventing its activation of its target genes [133]. Moreover, treatment of cultured MSCs with the HDAC inhibitor trichostatin A decreased adipogenic differentiation [112,134].

While it has been shown that some epigenetic modifiers act by direct activation or repression of *PPAR-γ2*, other modifiers such as EZH2 regulate adipogenesis indirectly through the inhibition of signaling pathways that promote osteo/chondrogenesis [115]. Furthermore, the recruitment of epigenetic modifiers to the chromatin by other molecules can act as another level of control to further regulate the expression of adipose-associated genes.

Collectively, these studies depict the complexity of epigenetic modifications involved in adipogenic differentiation, where various modifiers appear to be pivotal for the switch between osteogenic and adipogenic lineage commitment (Table 6.1).

## 6.7 Epigenetic regulation of myogenic differentiation

DNA methylation has been shown to be important for gene silencing during MSC differentiation. However, studies have found that the known myogenic differentiation 1 MyoD1 CpG islands are not methylated in many nonexpressing tissues, such as the brain [135]. These findings demonstrated that, in some instances, the CpG methylation status of genes did not dictate whether these genes were silenced or expressed, suggesting that other regulator mechanisms are required.

Other studies have shown that protein levels of the DNA methyl binding factors MeCP2 and MBD-2 significantly increased during myogenic differentiation and they are known to regulate chromatin reorganization and promotion of terminal differentiation [136]. While non-muscle-derived MSC-like populations do not normally form myotubes or constitutively express MYOD1 during culture, treatment with 5-azacytidine, which removes DNA methylation, was found to induce myosin heavy chain expression and myotube formation in these cells, and promoted skeletal muscle differentiation [137]. Furthermore, reports have described the localization of EZH2, YY1, and HDAC1 on silent muscle-specific genes in different MSCs-like populations, preventing gene muscle creatine kinase (MCK) and myosin heavy chain (MHC) transcription [138,139]. During muscle differentiation, EZH2, Y11, and HDAC1 are removed from muscle genes, allowing the recruitment of MYOD and serum response factor (SRF) to chromatin [138,140]. EZH2 has also been found to repress the microRNA miR-214 in MSCs, where EZH2 is removed from the chromatin during myogenic induction, allowing activation of miR-214 and activation of *MYOD* and *MYOGENIN* [141]. (MYOD is a protein with a key role in regulating muscle differentiation. Myogenin is a muscle-specific basic-helix–loop–helix (bHLH) transcription factor involved in the coordination of skeletal muscle development (myogenesis) and repair.)

The role of H3K27me HDMs in MSC differentiation was first described in muscle differentiation, where recruitment of UTX by the homeodomain transcription factor Six4 during myogenesis resulted in H3K27 demethylation of muscle-specific genes, RNA polymerase elongation, and gene expression [142]. In another study, MLL5, which lacks methyltransferase H3K4 activity like its family members MLL3 and MLL4, is critical for the regulation of LSD1 and SET7/9 and the expression of muscle regulators paired box 7 (PAX7), myogenic factor 5 (Myf5), and MYOGENIN [143]. However, the role of these factors in MSC skeletal muscle differentiation remains to be determined.

Collectively, studies suggest that epigenetic modifiers may play a role in regulating lineage-specific genes in myogenesis. However, further investigations are needed to determine which modifiers are critical for driving myogenic lineage-commitment in muscle- and non-muscle-derived MSC-like populations.

## 6.8 Epigenetic regulation of chondrogenic differentiation

Early studies showed that collagen type I is methylated in fibroblasts and chondrocytes, while the cartilage-specific collagen type II gene exhibits reduced methylation in chondrocytes compared with fibroblasts. In human chondrocytes the CpG sites within the COL10A1 promoter are normally methylated; however, during MSC chondrogenic differentiation *in vitro*, two CpG regions within the COL10A1 promoter undergo demethylation [144]. In another report, DNA hypermethylation of COL9A1 downregulated its expression in osteoarthritic chondrocytes, while treatment with 5-azadeoxycytidine increased

**Table 6.1** Histone modifiers that regulate MSC osteogenic and adipogenic differentiation.

| Epigenetic modifier | Modification | Osteogenesis | References | Adipogenesis | References |
|---|---|---|---|---|---|
| *DNA methylation* | | | | | |
| DMT1 | 5MC | Represses Runx2 and Alx5 in ADSCs.<br>Regulates ALP, Trip10, and RANKL in MSC. | [74–77] | DNA hypomethylation on PPARγ2 and Leptin in cultured adipose-derived MSC. | [118] |
| *Methylation* | | | | | |
| EZH2 | H3K27me3 | CDK1 phosphorylates EZH2, promoting osteogenesis. Overexpression repressed RUNX2 and OP expression. Ezh2 represses PPARγ2, promoting osteogenesis. | [87–89] | EZH2 regulates/represses noncanonical pathway promoting adipogenesis. Overexpression promotes adipogenesis. | [87,115] |
| Setdb1 | H3K9me3 | Suppresses PPARγ2 activation by H3K9me3. | [119,120] | Downregulated during adipogenesis. | [119,120] |
| Suv39h1 | H3K9me3 | — | | Suv39h1 mediates A2 repression of C/EBP-α | [117] |
| G9a | H3K9me3 | — | | Inhibition promotes PPARγ2 expression. | [122] |
| MLL3/MLL4 | H3K4me3 | Promotes osteogenesis by HOXA-mediated activation of Runx2 and OC. | [100] | Recruited to PPARγ2-activated gene *ap2*. | [99,123,125,126] |
| PR-Set7/Setd8 | H4K20me1 | — | | Overexpression promotes adipogenesis with methylation of PPARγ2. | [116] |
| PRMT5 | H3R81/2 | — | | Knockdown inhibits adipogenesis. | [130] |
| *Demethylation* | | | | | |
| UTX/KDM6A | H3K27me3 | Overexpression promotes osteogenesis with the demethylation of RUNX2 and OP. | [87] | Overexpression inhibits adipogenesis. | [87] |
| JMJD3/KDMB | H3K27me3 | Overexpression promotes RUNX2 and OSX expression and osteogenesis. | [90,91,93] | Knockdown-promoted adipogenesis. | [90] |
| JARIDIA | H3K4me3 | Inhibits RUNX2, ALP, and OSX by demethylation. | [101] | — | |
| KDM4B | H3K9me3 | Demethylates HOX and DLX promoting osteogenesis. | [90] | Knockdown-promoted adipogenesis. | [90] |
| LSD1 | H3K9me3 | — | | Promotes adipogenesis by removing H3K9me3 on C/EBPα. | [98] |
| N066 | H3K4me3 | Prevents OSX activation. | [102] | — | |
| RBP2 | H3K4me3 | Knockdown of RBP2 increased ALP, OC, and OSX expression. | [101] | — | |

(*continued*)

**Table 6.1** (*Continued*)

| Epigenetic modifier | Modification | Osteogenesis | References | Adipogenesis | References |
|---|---|---|---|---|---|
| *Acetylation* | | | | | |
| HATs | H3K9ac, H3K14ac | Dysregulation leads to osteogenesis in long-term cultured MSC. | [103] | — | |
| | H3ac, H4ac | Hyperacetylation prevents HDAC removal on OC and OSX. | [104–106] | — | |
| | | Regulates osteogenesis through Runx2. | [107] | — | |
| *HDAC inhibitors* | | | | | |
| TSA, vorinostat, sodium butyrate, valproic acid | H3ac, H4ac | Promoted osteogenesis. | [109–114] | Inhibited adipogenic differentiation. | [134] |
| *Deacetylases* | | | | | |
| HDAC | H3ac, H4ac | | | RB recruits HDAC3 prevents PPARγ2 activation. | [133] |

H3K4me3: histone three lysine 4 trimethylation; H3K27me3: histone 3 lysine 27 trimethylation; H3K9me3: histone 3 lysine 9 trimethylation; H4K20me2: histone 4 lysine20 monomethylation; H3K9ac: histone 3 lysine 9 acetylation; H3ac and H4ac: histone 3 and 4 acetylation; HDAC: histone deacetylases; HATs: histone acetyltransferases; DNMT1: DNA methyltransferases 1; Runx2: Runt-related transcription factor 2; OP: Osteopontin; OSX: Osterix; OC: Osteocalcin; RB: retinoblastoma; ALP: alkaline phosphatase; C/EBPα: CCAAT/enhancer-binding protein α; PPARγ2: peroxisome proliferator-activated receptor γ; HOX: homeotic genes; CDK1: cyclin-dependent kinase 1.

COL9A1 expression in these cells [145] and has previously been shown to induce chrondrogenesis [146]. In accord with these studies, the DNA methylation status of MSC chondrogenic differentiation was investigated, identifying eight key chondrogenic CpG-rich promoters – SOX9, RUNX2, the PAK kinase CHM1, fibroblast growth factor receptor (FGFR) 3, chondroadherin (CHAD), matrilin 4 (MATN4), SOX4, gremlin 1 (GREM1), and G-protein coupled receptor 39 (GPR39) – that were observed to be stable throughout differentiation [147]. These findings are consistent with previous reports suggesting that CpG-rich promoters remain stably hypomethylated in adult somatic cells, but not all methylation patterns are indicative of gene silencing or activation [148,149].

While studies examining the epigenetic regulation of chondrogenic differentiation of human MSCs are scant, one report showed that the chondrogenesis-specific gene collagen type II loses H3K27me3 and gains H3K4 and H3K36 active methylation during early chondrogenic induction [150]. In mice, chondrogenesis is regulated by Sox9 and its co-regulator AT-rich interactive domain 5b, which recruits the H3K9 demethylase plant homeodomain zinc finger-like 2 (Phf2) to the Sox9 promoter to reduce H3K29me2 levels and promote chondrogenesis [151].

In other studies the acetylase Tat interactive protein-60 (Tip60) can activate Sox9 and histone 4 (H4) acetylation, promoting Sox9-mediated transcription of COL2A1 [152]. Several other transcription factors and co-activators interact directly with Sox9 to modulate Sox9 activity. For example, The Sox9-dependent transcription of cartilage-associated target genes is thought to act via p300-mediated-histone acetylation on chromatin [153]. Consistent with these findings, the HDAC inhibitor trichostatin A was shown to stimulate the expression of Sox9-regulated cartilage matrix genes and induced histone acetylation around the enhancer region of the collagen alpha 1 (II) gene in chondrocytes [154,155]. Therefore, it appears that Sox9 interacts with chromatin and activates transcription via regulation of chromatin modification. Other studies have demonstrated that HDAC activity promotes type II collagen expression in chondrocytes by suppressing the transcription of *WNT-5A* [156]. Furthermore, overexpression of HDAC4 in synovium-derived MSCs was found to promote chondrogenesis in the presence of TGF-β1 [157], and is thought to also function by inhibiting RUNX2 activity [158].

With limited studies using MSC-derived chondrocytes, the impact of epigenetic modifiers on regulating chondrogenic differentiation is unclear. Collectively, these studies have shown that epigenetic modifiers play a role in articular cartilage and synovium-derived chondrocyte development.

This chapter has identified the role of epigenetic modifiers driving MSC lineage commitment. It has identified the direct regulation of key transcription factors RUNX2, SOX9, PPAR-γ2, and MYOD for MSC differentiation as targets of epigenetic modifiers (Table 6.2). These findings suggest a significant level of complexity driving MSC lineage determination.

**Table 6.2** Epigenetic modifiers regulate DNA and histone marks on master regulatory genes during MSC differentiation.

| Modification | PPARγ2 | RUNX2 | MYOD | SOX9 |
|---|---|---|---|---|
| 5-Methyl cytosine | Hypomethylation during adipogenesis [118] | Demethylation during osteogenesis [74] | Unmethylated in nonexpressing tissues [138] | — |
| H3K27me3 | Low methylation present in preadipocytes [115] | Methylated in MSCs [87,88]<br>Removed on osteoblasts [87,91] | Prevents MYOD recruitment to muscle genes [135]<br>miRNA removes EZH2 from MYOD in muscle differentiation [138,140] | — |
| H3K9me3 | Methylation on PPARγ2 promotes osteogenesis [96]<br>Inhibits adipogenesis. Methylation present in preadipocytes [115] | — | | Demethylation activates SOX9 during chondrogenesis [150,151] |
| H3K4me2 | Promotes adipogenesis [98,99,125,126] | Promotes osteogenesis. Methylation removed in adipocytes [100] | — | — |
| H4K20me1 | Promotes adipogenesis [116] | — | | — |
| H3R8 | Promotes adipogenesis [128] | — | | — |
| H3K9ac | Prevents PPARγ2 activation [133] | Dysregulation in MSCs lead to spontaneous osteogenic differentiation [103]<br>HDAC4 inhibits RUNX2, promoting chondrogenesis [157] | | Acetylases inhibition promotes chondrogenesis [154] |

H3K4me3: histone 3 lysine 4 trimethylation; H3K27me3: histone 3 lysine 27 trimethylation; H3K9me3: histone 3 lysine 9 trimethylation; H4K20me2: histone 4 lysine 20 monomethylation; H3K9ac: histone 3 lysine 9 acetylation; HDAC: histone deacetylase; *RUNX2*: Runt-related transcription factor 2; *PPARγ2*: peroxisome proliferator-activated receptor γ; *MYOD*: myogenic differentiation antigen; *SOX9*: SRY-related HMG-box 9.

## 6.9 Epigenetic regulation of mesenchymal stem/stromal cell lifespan and senescence

MSCs have a limited growth and differentiation potential when propagated *ex vivo*. DNA methylation plays critical roles in not only MSC differentiation but also in regulating the lifespan of MSCs. DNA methylation profiles of late-passage senescent BM-MSCs and adipose-derived MSCs revealed highly consistent modifications at specific CpG sites. These DNA methylation changes have been correlated with histone marks of previously published data sets, such as trimethylation of H3K9, H3K27, and EZH2 targets [159]. However, the reduced adipogenic differentiation capacity of late-passage senescent MSCs cannot be attributed to DNA methylation of adipogenic gene promoters alone [71]. Maharia and colleagues identified the role of 10-11-translocation 1/2 (TET1/2) DNA demethylases in regulating the DNA demethylation of insulin-like growth factor-binding protein 1. These findings suggest that TET1/2 regulates MSC self-renewal and proliferation [160].

EZH2 is important for senescence [159], where Twist-1 induces EZH2, directly or indirectly, which in turn suppresses the Ink4A/Arf locus and p16/p14, preventing senescence of human MSCs [161]. (ArF is an abbreviation for alternate reading frame; p16 Ink4A is named after its molecular weight and its role in inhibiting cyclin-dependent kinase 4A.) By analogy, EZH2 is also involved in preventing hematopoietic exhaustion and stem cell maintenance [162]. Spontaneous transformation of rat-derived MSCs has been attributed to the increase of histone H3K27me and H3K9me on the *p16 INK4a* gene and increased DNA methylation [163]. Furthermore, the replicative senescence of MSCs mediated by DNA methylation has been associated more with H3K27me histone marks rather than bivalent domains [159], meaning they have lost the activating H3K4me3 mark. (Bivalent chromatin is chromatin that contains both activating and repressing epigenetic modifications in the same area.) This is in contrast to differentiation of nonsenescent cells, which show many bivalent genes to have increased H3K4me3 levels and decreased H3K27me3 levels while being transcriptionally activated during differentiation [66,68–72,164]. During long-term culture, MSCs undergo spontaneous osteogenic differentiation associated with downregulation of telomerase reverse transcriptase and the self-renewal genes *OCT4* and *SOX2* and an upregulation of osteogenic genes.

DNA methylation and histone modification play a role in dictating MSC lifespan and senescence. It is important to understand how these modifiers can influence the lifespan of MSCs, since this may aid in prolonging the survival and expansion capacity of MSCs. Extending the *in vitro* expansion capacity of MSCs would be highly beneficial for manufacturing MSCs for cell-based therapies.

## 6.10 Regulation of epigenetic modifications in mesenchymal stem/stromal cells for clinical use

Following on from the initial human safety trials in 1995 [165], both allogenic and autologous MSC preparations have now been used to treat patients with conditions such as graft-versus-host disease, cardiovascular diseases, neurologic diseases, autoimmune diseases, organ transplantation, refractory wounds, and bone and cartilage defects [166,167]. Continued investigations into the molecular mechanisms that regulate MSC development, self-renewal, and proliferation will likely help develop optimal cell expansion protocols and quality control criteria to overcome the features currently associated with MSCs following *ex vivo* expansion. These include donor variability, diminishing functionality, and limited lifespan. Therefore, a better understanding of the epigenetic modifications, which regulate the growth and multipotential capability of MSCs, could be used in strategies to manipulate MSC populations *ex vivo*. The application of chemical and pharmaceutical agents that regulate epigenetic modifiers may be beneficial in priming MSCs for specific therapies and could potentially be incorporated into scaffold and cell delivery systems. However, potential therapeutic strategies, such as the use of inhibitor molecules, have to await the development of novel agents that target specific epigenetic modifiers in order to maximize tissue regenerative ability with minimal side-effects.

## 6.11 Conclusions

This chapter has summarized the role of epigenetic modifiers in MSC differentiation, lifespan, and senescence. The presence of repressive DNA methylation, H3K27me3, H3K9me3, and HDACs on the promoter regions of key genes and signaling pathways often prevents adipogenic, osteogenic, chrondrogenic, and myogenic differentiation of MSCs, whereas the presence of DNA demethylases H3K4me3, H3K20me1, and HATs on the promoter regions of key genes and signaling pathways can facilitate adipogenic, osteogenic, chondrogenic, and myogenic differentiation of MSCs. Ultimately, the histone/DNA modifications lead to recruitment of chromatin remodelers which results in gene activation or repression. While some chromatin remodelers have been identified during osteogenic, adipogenic, chondrogenic, and myogenic differentiation, it is still unclear how and which posttranslational modifications work together to determine the activation and repression of genes important for MSC lineage commitment and lifespan.

With the use of genome-wide sequencing techniques such as ChIP-seq, RNA-seq, ChIP-on-chip, and microarray, future studies may be able to correlate the epigenetic patterning during many cellular events, including MSC multidifferentiation ability and growth to aid in the development of more potent MSC preparations for regenerative medicine applications. It is also

hoped that increased knowledge in this field may lead to the identification of epigenetic marks associated with impaired MSC growth and function associated with different musculoskeletal diseases, such as osteoporosis and osteoarthritis, in order to develop better prognostic criteria and novel treatment regimes.

## References


1 Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411): 143–147.

2 Rogers I, Casper RF. Umbilical cord blood stem cells. *Best Pract Res Clin Obstet Gynaecol* 2004;**18**(6):893–908.

3 Bieback K, Kluter H. Mesenchymal stromal cells from umbilical cord blood. *Curr Stem Cell Res Ther* 2007;**2**(4):310–323.

4 Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003;**18**(4):696–704.

5 Igura K, Zhang X, Takahashi K, *et al.* Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy* 2004;**6**(6):543–553.

6 De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001;**44**(8):1928–1942.

7 Schipani E, Kronenberg HM. Adult mesenchymal stem cells. In *StemBook [Internet]*, The Stem Cell Research Community (eds). Harvard Stem Cell Institute: Cambridge, MA, 2009.

8 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

9 Gronthos S, Mankani M, Brahim J, *et al.* Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2000;**97**:13625–13630.

10 Seo BM, Miura M, Gronthos S, *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* 2004;**364**(9429):149–155.

11 Kuznetsov SA, Mankani MH, Gronthos S, *et al.* Circulating skeletal stem cells. *J Cell Biol* 2001;**153**(5):1133–1140.

12 Li MH, Tian D, Liu CY, *et al.* Expansion *ex vivo* of human bone marrow mesenchymal stem cells and cord blood $CD34^+$ cells. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2005;**13**(2):235–239 (in Chinese).

13 Bi Y, Ehirchiou D, Kilts TM, *et al.* Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;**13**(10):1219–1227.

14 Friedenstein AJ, Chailakh RK, Lalykina KS. Development of fibroblast colonies in monolayer culture of guinea-pig bone-marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**(4):393–404.

15 Castromalaspina H, Gay RE, Resnick G, *et al.* Characterization of human bone-marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* 1980;**56**(2):289–301.

16 Gronthos S, Zannettino ACW, Hay SJ, *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003;**116**(9):1827–1835.

17 Kuznetsov SA, Friedenstein AJ, Robey PG. Factors required for bone marrow stromal fibroblast colony formation *in vitro*. *Br J Haematol* 1997;**97**(3):561–570.

18 Sacchetti B, Funari A, Michienzi S, *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**(2):324–336.

19 Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* 2000;**113**(7):1161–1166.

20 Owen M, Friedenstein AJ. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988;**136**:42–60.

21 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

22 Patel DM, Shah J, Srivastava AS. Therapeutic potential of mesenchymal stem cells in regenerative medicine. *Stem Cells Int* 2013;**2013**:496218.

23 Zannettino ACW, Paton S, Kortesidis A, *et al.* Human multipotential mesenchymal/stromal stem cells are derived from a discrete subpopulation of STRO-$1^{bright}$/$CD34^-$/$CD45^-$/glycophorin-A-bone marrow cells. *Haematologica* 2007;**92**(12):1707–1708.

24 Gronthos S, Zannettino AC. A method to isolate and purify human bone marrow stromal stem cells. *Methods Mol Biol* 2008;**449**:45–57.

25 Isenmann S, Arthur A, Zannettino ACW, *et al.* TWIST family of basic helix–-loop–helix transcription factors mediate human mesenchymal stem cell growth and commitment. *Stem Cells* 2009;**27**(10):2457–2468.

26 Menicanin D, Barfold PM, Zannettino AC, Gronthos S. Identification of a common gene expression signature associated with immature clonal mesenchymal cell populations derived from bone marrow and dental tissues. *Stem Cells Dev* 2010;**19**:1501–1510.

27 Kortesidis A, Zannettino A, Isenmann S, *et al.* Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. *Blood* 2005;**105**(10):3793–3801.

28 Lin GL, Hankenson KD. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. *J Cell Biochem* 2011;**112**(12):3491–3501.

29 Holliday R. Epigenetics: an overview. *Dev Genet* 1994;**15**(6):453–457.

30 Luger K, Rechsteiner TJ, Flaus AJ, *et al.* Characterization of nucleosome core particles containing histone proteins made in bacteria. *J Mol Biol* 1997;**272**(3):301–311.

31 Luger K, Mader AW, Richmond RK, *et al.* Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* 1997; **389**(6648):251–260.

32 Riggs AD. X inactivation, differentiation, and DNA methylation. *Cytogenet Cell Genet* 1975;**14**(1):9–25.

33 Holliday R, Pugh JE. DNA modification mechanisms and gene activity during development. *Science* 1975;**187**(4173):226–232.

34 Miniou P, Jeanpierre M, Blanquet V, *et al.* Abnormal methylation pattern in constitutive and facultative (X inactive chromosome) heterochromatin of ICF patients. *Hum Mol Genet* 1994;**3**(12): 2093–2102.

35 Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet* 1998;**19**(3):219–220.

36 Bird A. DNA methylation patterns and epigenetic memory. *Genes Dev* 2002;**16**(1):6–21.

37 Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;**33**(3):245–254.

38 Li E, Beard C, Jaenisch R. Role for DNA methylation in genomic imprinting. *Nature* 1993;**366**(6453):362–365.

39 Caspary T, Cleary MA, Baker CC, *et al.* Multiple mechanisms regulate imprinting of the mouse distal chromosome 7 gene cluster. *Mol Cell Biol* 1998;**18**(6):3466–3474.

40 Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet* 2002;**3**(9): 662–673.

41 Shock LS, Thakkar PV, Peterson EJ, *et al.* DNA methyltransferase 1, cytosine methylation, and cytosine hydroxymethylation in mammalian mitochondria. *Proc Natl Acad Sci U S A* 2011;**108**(9): 3630–3635.

42 Ulaner GA, Vu TH, Li T, *et al.* Loss of imprinting of IGF2 and H19 in osteosarcoma is accompanied by reciprocal methylation changes of a CTCF-binding site. *Hum Mol Genet* 2003;**12**(5):535–549.

43 Razin A, Shemer R. DNA methylation in early development. *Hum Mol Genet* 1995;**4**: Spec No. 1751-5.

44 Monk M, Boubelik M, Lehnert S. Temporal and regional changes in DNA methylation in the embryonic, extraembryonic and germ cell lineages during mouse embryo development. *Development* 1987;**99** (3):371–382.

45 Kafri T, Ariel M, Brandeis M, *et al.* Developmental pattern of gene-specific DNA methylation in the mouse embryo and germ line. *Genes Dev* 1992;**6**(5):705–714.

46 Robertson KD. DNA methylation, methyltransferases and cancer. *Oncogene* 2001;**20**(24):3139–3155.

47 Vaissiere T, Sawan C, Herceg Z. Epigenetic interplay between histone modifications and DNA methylation in gene silencing. *Mutat Res* 2008;**659**(1–2):40–48.

48 Antequera F. Structure, function and evolution of CpG island promoters. *Cell Mol Life Sci* 2003;**60**(8):1647–1658.

49 Ballestar E, Wolffe AP. Methyl-CpG-binding proteins. Targeting specific gene repression. *Eur J Biochem* 2001;**268**(1):1–6.

50 El-Osta A, Wolffe AP. DNA methylation and histone deacetylation in the control of gene expression: basic biochemistry to human development and disease. *Gene Expr* 2000;**9**(1–2):63–75.

51 Fuks F, Hurd PJ, Wolf D, *et al.* The methyl-CpG-binding protein MeCP2 links DNA methylation to histone methylation. *J Biol Chem* 2003;**278**(6):4035–4040.

52 Wolffe AP, Matzke MA. Epigenetics: regulation through repression. *Science* 1999;**286**(5439):481–486.

53 Wu H, Zhang Y. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev* 2011;**25**(23): 2436–2452.

54 Tahiliani M, Koh KP, Shen Y, *et al.* Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009;**324**(5929):930–935.

55 Warnecke PM, Stirzaker C, Song J, *et al.* Identification and resolution of artifacts in bisulfite sequencing. *Methods* 2002;**27**(2):101–107.

56 Grunau C, Clark SJ, Rosenthal A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res* 2001;**29**(13):E65–5.

57 Bock C, Beerman I, Lien WH, *et al.* DNA methylation dynamics during *in vivo* differentiation of blood and skin stem cells. *Mol Cell* 2012;**47**(4):633–647.

58 Smith ZD, Meissner A. DNA methylation: roles in mammalian development. *Nat Rev Genet* 2013;**14**(3):204–220.

59 Mikkelsen TS, Xu Z, Zhang X, *et al.* Comparative epigenomic analysis of murine and human adipogenesis. *Cell* 2010;**143**(1): 156–169.

60 Fisher AG. Cellular identity and lineage choice. *Nat Rev Immunol* 2002;**2**(12):977–982.

61 Bernstein BE, Meissner A, Lander ES. The mammalian epigenome. *Cell* 2007;**128**(4):669–681.

62 Bernstein B, Mikkelsen T, Xie X, *et al.* A bivalent chromatin structure marks key developmental genes in embryonic stem cells. *Cell* 2006;**125**(2):315–326.

63 Sasai N, Defossez PA. Many paths to one goal? The proteins that recognize methylated DNA in eukaryotes. *Int J Dev Biol* 2009; **53**(2–3):323–334.

64 Taverna SD, Li H, Ruthenburg AJ, *et al.* How chromatin-binding modules interpret histone modifications: lessons from professional pocket pickers. *Nat Struct Mol Biol* 2007;**14**(11): 1025–1040.

65 Teven CM, Liu X, Hu N, *et al.* Epigenetic regulation of mesenchymal stem cells: a focus on osteogenic and adipogenic differentiation. *Stem Cells Int* 2011;**2011**:201371.

66 Sørensen AL, Timoskainen S, West FD, *et al.* Lineage-specific promoter DNA methylation patterns segregate adult progenitor cell types. *Stem Cells Dev* 2010;**19**(8):1257–1266.

67 Sørensen AL, Jacobsen BM, Reiner AH, *et al.* Promoter DNA methylation patterns of differentiated cells are largely programmed at the progenitor stage. *Mol Biol Cell* 2010;**21**(12): 2066–2077.

68 Collas P. Programming differentiation potential in mesenchymal stem cells. *Epigenetics* 2010;**5**(6):476–482.

69 Kang MI, Kim HS, Jung YC, *et al.* Transitional CpG methylation between promoters and retroelements of tissue-specific genes during human mesenchymal cell differentiation. *J Cell Biochem* 2007;**102**(1):224–239.

70 Noer A. Stable CpG hypomethylation of adipogenic promoters in freshly isolated, cultured, and differentiated mesenchymal stem cells from adipose tissue. *Mol Biol Cell* 2006;**17**(8): 3543–3556.

71 Noer A, Boquest AC, Collas P. Dynamics of adipogenic promoter DNA methylation during clonal culture of human adipose stem cells to senescence. *BMC Cell Biol* 2007;**8**:18.

72 Collas P, Noer A, Sørensen AL. Epigenetic basis for the differentiation potential of mesenchymal and embryonic stem cells. *Transfus Med Hemother* 2008;**35**(3):205–215.

73 Deng ZL, Sharff KA, Tang N, *et al.* Regulation of osteogenic differentiation during skeletal development. *Front Biosci* 2008;**13**: 2001–2021.

74 Zhang RP, Shao JZ, Xiang LX. GADD45A protein plays an essential role in active DNA demethylation during terminal osteogenic differentiation of adipose-derived mesenchymal stem cells. *J Biol Chem* 2011;**286**(47):41083–94.

75 Kitazawa R, Kitazawa S. Methylation status of a single CpG locus 3 bases upstream of TATA-box of receptor activator of nuclear factor-κB ligand (RANKL) gene promoter modulates cell- and tissue-specific RANKL expression and osteoclastogenesis. *Mol Endocrinol* 2007;**21**(1):148–158.

76 Delgado-Calle J, Sañudo C, Sánchez-Verde L, *et al.* Epigenetic regulation of alkaline phosphatase in human cells of the osteoblastic lineage. *Bone* 2011;**49**(4):830–838.

77 Hsiao S-H, Lee K-D, Hsu C-C, *et al.* DNA methylation of the *Trip10* promoter accelerates mesenchymal stem cell lineage determination. *Biochem Biophys Res Commun* 2010;**400**(3):305–312.

78 Dansranjavin T, Krehl S, Mueller T, *et al.* The role of promoter CpG methylation in the epigenetic control of stem cell related genes during differentiation. *Cell Cycle* 2009;**8**(6):916–924.

79 Arnsdorf EJ, Tummala P, Castillo AB, *et al.* The epigenetic mechanism of mechanically induced osteogenic differentiation. *J Biomech* 2010;**43**(15):2881–2886.

80 Vire E, Brenner C, Deplus R, *et al.* The Polycomb group protein EZH2 directly controls DNA methylation. *Nature* 2006; **439**(7078):871–874.

81 Chambeyron S, Bickmore WA. Chromatin decondensation and nuclear reorganization of the HoxB locus upon induction of transcription. *Genes Dev* 2004;**18**(10):1119–1130.

82 Perry P, Sauer S, Billon N, *et al.* A dynamic switch in the replication timing of key regulator genes in embryonic stem cells upon neural induction. *Cell Cycle* 2004;**3**(12):1645–1650.

83 Francis NJ, Kingston RE, Woodcock CL. Chromatin compaction by a polycomb group protein complex. *Science* 2004;**306**(5701): 1574–1577.

84 Ringrose L, Ehret H, Paro R. Distinct contributions of histone H3 lysine 9 and 27 methylation to locus-specific stability of polycomb complexes. *Mol Cell* 2004;**16**(4):641–653.

85 Ringrose L, Paro R. Epigenetic regulation of cellular memory by the polycomb and trithorax group proteins. *Annu Rev Genet* 2004;**38**:413–443.

86 Sparmann A, van Lohuizen M. Polycomb silencers control cell fate, development and cancer. *Nat Rev Cancer* 2006;**6**(11): 846–856.

87 Hemming S, Cakouros D, Isenmann S, *et al. EZH2* and *KDM6A* act as an epigenetic switch to regulate mesenchymal stem cell lineage specification. *Stem Cells* 2014;**32**(3):802–815.

88 Wei Y, Chen Y-H, Li L-Y, *et al.* CDK1-dependent phosphorylation of EZH2 suppresses methylation of H3K27 and promotes osteogenic differentiation of human mesenchymal stem cells. *Nat Cell Biol* 2010;**13**(1):87–94.

89 Chen YH, Yeh FL, Yeh SP, *et al.* Myocyte enhancer factor-2 interacting transcriptional repressor (MITR) is a switch that promotes osteogenesis and inhibits adipogenesis of mesenchymal stem cells by inactivating peroxisome proliferator-activated receptor-2. *J Biol Chem* 2011;**286**(12):10671–10680.

90 Ye L, Fan Z, Yu B, *et al.* Histone demethylases KDM4B and KDM6B promotes osteogenic differentiation of human MSCs. *Cell Stem Cell* 2012;**11**(1):50–61.

91 Yang D, Okamura H, Nakashima Y, Haneji T. Histone demethylase Jmjd3 regulates osteoblast differentiation via transcription factors Runx2 and Osterix. *J Biol Chem* 2013;**288**(47):33530–33541.

92 Huszar JM, Payne CJ. MIR146A inhibits JMJD3 expression and osteogenic differentiation in human mesenchymal stem cells. *FEBS Lett* 2014;**588**(9):1850–1856.

93 Xu J, Yu B, Hong C, Wang C-Y. KDM6B epigenetically regulates odontogenic differentiation of dental mesenchymal stem cells. *Int J Oral Sci* 2013;**5**(4):200–205.

94 Wu H, Sun YE. Epigenetic regulation of stem cell differentiation. *Pediatr Res* 2006;**59**(4 Pt 2):21R–25R.

95 Tan J, Lu J, Huang W, *et al.* Genome-wide analysis of histone H3 lysine9 modifications in human mesenchymal stem cell osteogenic differentiation. *PLoS ONE* 2009;**4**(8):e6792.

96 Burdge GC, Lillycrop KA. Bridging the gap between epigenetics research and nutritional public health interventions. *Genome Med* 2010;**2**(11):80.

97 Santos-Rosa H, Schneider R, Bernstein BE, *et al.* Methylation of histone H3K4 mediates association of the Isw1p ATPase with chromatin. *Mol Cell* 2003;**12**(5):1325–1332.

98 Musri MM, Corominola H, Casamitjana R, *et al.* Histone H3 lysine 4 dimethylation signals the transcriptional competence of the adiponectin promoter in preadipocytes. *J Biol Chem* 2006;**281** (25):17180–17188.

99 Cho YW, Hong T, Hong S, *et al.* PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. *J Biol Chem* 2007;**282**(28):20395–20406.

100 Hassan MQ, Tare R, Lee SH, *et al.* HOXA10 controls osteoblastogenesis by directly activating bone regulatory and phenotypic genes. *Mol Cell Biol* 2007;**27**(9):3337–3352.

101 Ge W, Shi L, Zhou Y, *et al.* Inhibition of osteogenic differentiation of human adipose-derived stromal cells by retinoblastoma binding protein 2 repression of RUNX2-activated transcription. *Stem Cells* 2011;**29**(7):1112–1125.

102 Sinha KM, Yasuda H, Coombes MM, *et al.* Regulation of the osteoblast-specific transcription factor Osterix by NO66, a Jumonji family histone demethylase. *Embo J* 2010;**29**(1):68–79.

103 Li Z, Liu C, Xie Z, *et al.* Epigenetic dysregulation in mesenchymal stem cell aging and spontaneous differentiation. *PLoS ONE* 2011; **6**(6):e20526.

104 Westendorf JJ, Zaidi SK, Cascino JE, *et al.* Runx2 (Cbfa1, AML-3) interacts with histone deacetylase 6 and represses the $p21^{CIP1/WAF1}$ promoter. *Mol Cell Biol* 2002;**22**(22):7982–7992.

105 Jensen ED, Schroeder TM, Bailey J, *et al.* Histone deacetylase 7 associates with Runx2 and represses its activity during osteoblast maturation in a deacetylation-independent manner. *J Bone Miner Res* 2008;**23**(3):361–372.

106 Schroeder TM, Kahler RA, Li X, Westendorf JJ. Histone deacetylase 3 interacts with runx2 to repress the osteocalcin promoter and regulate osteoblast differentiation. *J Biol Chem* 2004;**279**(40): 41998–42007.

107 McGee-Lawrence ME, Westendorf JJ. Histone deacetylases in skeletal development and bone mass maintenance. *Gene* 2011;**474** (1–2):1–11.

108 Lee TI, Jenner RG, Boyer LA, *et al.* Control of developmental regulator's by polycomb in human embryonic stem cells. *Cell* 2006;**125**(2):301–313.

109 Cho HH, Park HT, Kim YJ, *et al.* Induction of osteogenic differentiation of human mesenchymal stem cells by histone deacetylase inhibitors. *J Cell Biochem* 2005;**96**(3):533–542.

110 Lee HW, Suh JH, Kim AY, *et al.* Histone deacetylase 1-mediated histone modification regulates osteoblast differentiation. *Mol Endocrinol* 2006;**20**(10):2432–2443.

111 Xu S, De Veirman K, Evans H, *et al.* Effect of the HDAC inhibitor vorinostat on the osteogenic differentiation of mesenchymal stem cells *in vitro* and bone formation *in vivo*. *Acta Pharmacol Sin* 2013;**34**(5):699–709.

112 Lee S, Park JR, Seo MS, *et al.* Histone deacetylase inhibitors decrease proliferation potential and multilineage differentiation capability of human mesenchymal stem cells. *Cell Prolif* 2009;**42**(6):711–720.

113 De Boer J, Licht R, Bongers M, *et al.* Inhibition of histone acetylation as a tool in bone tissue engineering. *Tissue Eng* 2006;**12**(10): 2927–2937.

114 Di Bernardo G, Squillaro T, Dell'Aversana C, *et al.* Histone deacetylase inhibitors promote apoptosis and senescence in human mesenchymal stem cells. *Stem Cells Dev* 2009;**18**(4):573–581.

115 Wang L, Jin Q, Lee JE, *et al.* Histone H3K27 methyltransferase Ezh2 represses Wnt genes to facilitate adipogenesis. *Proc Natl Acad Sci U S A* 2010;**107**(16):7317–7322.

116 Wakabayashi K, Okamura M, Tsutsumi S, *et al.* The peroxisome proliferator-activated receptor gamma/retinoid X receptor alpha heterodimer targets the histone modification enzyme PR-Set7/Setd8 gene and regulates adipogenesis through a positive feedback loop. *Mol Cell Biol* 2009;**29**(13):3544–3555.

117 Zhang ZC, Liu Y, Li SF, *et al.* Suv39h1 mediates AP-2α-dependent inhibition of C/EBPα expression during adipogenesis. *Mol Cell Biol* 2014;**34**(12):2330–2338. doi: 10.1128/MCB.00070-14.

118 Fu M, Rao M, Bouras T, *et al.* Cyclin D1 inhibits peroxisome proliferator-activated receptor γ-mediated adipogenesis through histone deacetylase recruitment. *J Biol Chem* 2005;**280**(17):16934–16941.

119 Takada I, Mihara M, Suzawa M, *et al.* A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-γ transactivation. *Nat Cell Biol* 2007;**9**(11):1273–1285.

120 Takada I, Kouzmenko AP, Kato S. Wnt and PPARγ signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* 2009;**5**(8):442–447.

121 Wu H, Chen X, Xiong J, *et al.* Histone methyltransferase G9a contributes to H3K27 methylation *in vivo*. *Cell Res* 2010;**21**(2):365–367.

122 Wang L, Xu S, Lee JE, *et al.* Histone H3K9 methyltransferase G9a represses PPARγ expression and adipogenesis. *Embo J* 2013;**32**(1):45–59.

123 Musri MM, Carmona MC, Hanzu FA, *et al.* Histone demethylase LSD1 regulates adipogenesis. *J Biol Chem* 2010;**285**(39):30034–30041.

124 Musri MM, Gomis R, Parrizas M. A chromatin perspective of adipogenesis. *Organogenesis* 2010;**6**(1):15–23.

125 Lee JE, Wang C, Xu S, *et al.* H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation. *Elife (Cambridge)* 2013;**2**:e01503.

126 Lee J-E, Ge K. Transcriptional and epigenetic regulation of PPARγ expression during adipogenesis. *Cell Biosci* 2014;**4** (1): 29.

127 Okamura M, Inagaki T, Tanaka T, Sakai J. Role of histone methylation and demethylation in adipogenesis and obesity. *Organogenesis* 2010;**6**(1):24–32.

128 Bedford MT, Richard S. Arginine methylation an emerging regulator of protein function. *Mol Cell* 2005;**18**(3):263–272.

129 LeBlanc SE, Konda S, Wu Q, *et al.* Protein arginine methyltransferase 5 (Prmt5) promotes gene expression of peroxisome proliferator-activated receptor γ2 (PPARγ2) and its target genes during adipogenesis. *Mol Endocrinol* 2012;**26**(4):583–597.

130 Salma N, Xiao H, Mueller E, Imbalzano AN. Temporal recruitment of transcription factors and SWI/SNF chromatin-remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor gamma nuclear hormone receptor. *Mol Cell Biol* 2004;**24**(11):4651–4663.

131 Johmura Y, Osada S, Nishizuka M, Imagawa M. FAD24 acts in concert with histone acetyltransferase HBO1 to promote adipogenesis by controlling DNA replication. *J Biol Chem* 2008;**283**(4):2265–2274.

132 Kim EY, Kim WK, Kang HJ, *et al.* Acetylation of malate dehydrogenase 1 promotes adipogenic differentiation via activating its enzymatic activity. *J Lipid Res* 2012;**53**(9):1864–1876.

133 Fajas L, Egler V, Reiter R, *et al.* The retinoblastoma-histone deacetylase 3 complex inhibits PPARγ and adipocyte differentiation. *Dev Cell* 2002;**3**(6):903–910.

134 Zych J, Stimamiglio MA, Senegaglia AC, *et al.* The epigenetic modifiers 5-aza-2′-deoxycytidine and trichostatin A influence adipocyte differentiation in human mesenchymal stem cells. *Braz J Med Biol Res* 2013;**46**(5):405–416.

135 Jones PA, Wolkowicz MJ, Rideout WM, III, *et al. De novo* methylation of the MyoD1 CpG island during the establishment of immortal cell lines. *Proc Natl Acad Sci U S A* 1990;**87**(16):6117–6121.

136 Brero A. Methyl CpG-binding proteins induce large-scale chromatin reorganization during terminal differentiation. *J Cell Biol* 2005;**169**(5):733–743.

137 Nakatsuka R, Nozaki T, Uemura Y, *et al.* 5-Aza-2′-deoxycytidine treatment induces skeletal myogenic differentiation of mouse dental pulp stem cells. *Arch Oral Biol* 2010;**55**(5):350–357.

138 Caretti G, Di Padova M, Micales B, *et al.* The Polycomb Ezh2 methyltransferase regulates muscle gene expression and skeletal muscle differentiation. *Genes Dev* 2004;**18**(21):2627–2638.

139 Van der Vlag J, Otte AP. Transcriptional repression mediated by the human polycomb-group protein EED involves histone deacetylation. *Nat Genet* 1999;**23**(4):474–478.

140 Mal A, Harter ML. MyoD is functionally linked to the silencing of a muscle-specific regulatory gene prior to skeletal myogenesis. *Proc Natl Acad Sci U S A* 2003;**100**(4):1735–1739.

141 Juan AH, Kumar RM, Marx JG, *et al.* Mir-214-dependent regulation of the polycomb protein Ezh2 in skeletal muscle and embryonic stem cells. *Mol Cell* 2009;**36**(1):61–74.

142 Seenundun S, Rampalli S, Liu Q-C, *et al.* UTX mediates demethylation of H3K27me3 at muscle-specific genes during myogenesis. *EMBO J* 2010;**29**(8):1401–1411.

143 Sebastian S, Sreenivas P, Sambasivan R, *et al.* MLL5, a trithorax homolog, indirectly regulates H3K4 methylation, represses cyclin A2 expression, and promotes myogenic differentiation. *Proc Natl Acad Sci U S A* 2009;**106**(12):4719–4724.

144 Zimmermann P, Boeuf S, Dickhut A, *et al.* Correlation of COL10A1 induction during chondrogenesis of mesenchymal stem cells with demethylation of two CpG sites in the COL10A1 promoter. *Arthritis Rheum* 2008;**58**(9):2743–2753.

145 Imagawa K, de Andres MC, Hashimoto K, *et al.* Association of reduced type IX collagen gene expression in human osteoarthritic chondrocytes with epigenetic silencing by DNA hypermethylation. *Arthritis Rheumatol* 2014;**66**(11):3040–3051.

146 Studer D, Millan C, Ozturk E, *et al.* Molecular and biophysical mechanisms regulating hypertrophic differentiation in chondrocytes and mesenchymal stem cells. *Eur Cell Mater* 2012;**24**:118–135.

147 Ezura Y, Sekiya I, Koga H, *et al.* Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. *Arthritis Rheumatism* 2009;**60**(5):1416–1426.

148 Mohn F, Weber M, Rebhan M, *et al.* Lineage-specific polycomb targets and *de novo* DNA methylation define restriction and potential of neuronal progenitors. *Mol Cell* 2008;**30**(6):755–766.

149 Meissner A, Mikkelsen TS, Gu H, *et al.* Genome-scale DNA methylation maps of pluripotent and differentiated cells. *Nature* 2008;**454**(7205):766–770.

150 Herlofsen SR, Bryne JC, Hoiby T, *et al.* Genome-wide map of quantified epigenetic changes during *in vitro* chondrogenic differentiation of primary human mesenchymal stem cells. *BMC Genomics* 2013;**14**:105.

151 Hata K, Takashima R, Amano K, *et al.* Arid5b facilitates chondrogenesis by recruiting the histone demethylase Phf2 to Sox9-regulated genes. *Nat Commun* 2013;**4**:2850.

152 Hattori T, Coustry F, Stephens S, *et al.* Transcriptional regulation of chondrogenesis by coactivator Tip60 via chromatin association with Sox9 and Sox5. *Nucleic Acids Res* 2008;**36**(9):3011–3024.

153 Furumatsu T, Ozaki T. Epigenetic regulation in chondrogenesis. *Acta Med Okayama* 2010;**64**(3):155–161.

154 Furumatsu T, Tsuda M, Yoshida K, *et al.* Sox9 and p300 cooperatively regulate chromatin-mediated transcription. *J Biol Chem* 2005;**280**(42):35203–35208.

155 El-Serafi AT, Oreffo RO, Roach HI. Epigenetic modifiers influence lineage commitment of human bone marrow stromal cells: differential effects of 5-aza-deoxycytidine and trichostatin A. *Differentiation* 2011;**81**(1):35–41.

156 Huh YH, Ryu JH, Chun JS. Regulation of type II collagen expression by histone deacetylase in articular chondrocytes. *J Biol Chem* 2007;**282**(23):17123–17131.

157 Pei M, Chen D, Li J, Wei L. Histone deacetylase 4 promotes TGF-β1-induced synovium-derived stem cell chondrogenesis but inhibits chondrogenically differentiated stem cell hypertrophy. *Differentiation* 2009;**78**(5):260–268.

158 Vega RB, Matsuda K, Oh J, *et al.* Histone deacetylase 4 controls chondrocyte hypertrophy during skeletogenesis. *Cell* 2004; **119**(4):555–566.

159 Schellenberg A, Lin Q, Schuler H, *et al.* Replicative senescence of mesenchymal stem cells causes DNA-methylation changes which correlate with repressive histone marks. *Aging (Albany NY)* 2011; **3**(9):873–888.

160 Mahaira LG, Katsara O, Pappou E, *et al.* IGF2BP1 expression in human mesenchymal stem cells significantly affects their proliferation and is under the epigenetic control of TET1/2 demethylases. *Stem Cells Dev* 2014;**23**(20):2501–2512.

161 Cakouros D, Isenmann S, Cooper L, *et al.* Twist-1 induces Ezh2 recruitment regulating histone methylation along the Ink4A/Arf locus in mesenchymal stem cells. *Mol Cell Biol* 2012;**32**(8): 1433–1441.

162 Kamminga LM, Bystrykh LV, de Boer A, *et al.* The Polycomb group gene *Ezh2* prevents hematopoietic stem cell exhaustion. *Blood* 2006;**107**(5):2170–2179.

163 Zheng Y, He L, Wan Y, Song J. H3K9me-enhanced DNA hypermethylation of the $p16^{INK4a}$ gene: an epigenetic signature for spontaneous transformation of rat mesenchymal stem cells. *Stem Cells Dev* 2013;**22**(2):256–267.

164 Easwaran H, Johnstone SE, Van Neste L, *et al.* A DNA hypermethylation module for the stem/progenitor cell signature of cancer. *Genome Res* 2012;**22**(5):837–849.

165 Lazarus HM, Haynesworth SE, Gerson SL, *et al. Ex vivo* expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplant* 1995;**16** (4):557–564.

166 Ikebe C, Suzuki K. Mesenchymal stem cells for regenerative therapy: optimization of cell preparation protocols. *BioMed Res Int* 2014;**2014**:951512 .

167 Lalu MM, McIntyre L, Pugliese C, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.

CHAPTER 7

# Biological changes in human mesenchymal stromal cells during monolayer culture

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## 7.1 Introduction

In addition to hematopoietic stem cells that give rise to the cells of the hematopoietic and immune systems, bone marrow also contains a second type of adult stem cell: the mesenchymal stem cell. This cell can both self-renew and differentiate to tissues of the skeleton, including bone, cartilage, and adipose tissue. These cells were first identified in bone marrow cultures of several mammalian species in the late 1960s by Friedenstein and co-workers, who cloned them and called the colonies that single cells gave rise to as colony-forming units–fibroblastic (CFU-Fs) because of their morphological similarity to fibroblasts [1,2]. The true mesenchymal stem cell is a rare cell in the bone marrow, its frequency being assessed at 1 cell in $10^4$ to 1 cell in $10^5$ of bone marrow mononuclear cells. The mesenchymal stem cell also differentiates to mesenchymal stromal cells (MSCs), which form part of the wall of blood vessels, which are sometimes known as mesenchymal progenitor cells and can be derived from all vascularized tissues [3]. The abbreviation "MSC" will be used in this chapter to denote mesenchymal stromal cell – one of the progeny of true mesenchymal stem cells.

Multiple human prenatal, postnatal, and adult tissues contain this population of stromal progenitors. MSCs have been extensively characterized as a progenitor/stromal cell population on the basis of their ability to differentiate along mesenchymal lineages *in vivo* [3] and *in vitro*. The potential of these cells to give rise to multiple cell types, including chondrocytes, osteoblasts, adipocytes, myocytes, and tenocytes, initiated an entire field of research focused on harnessing the regenerative capacity of these cells to repair or engineer musculoskeletal tissues.

With regard to adult tissues, MSCs have been isolated from many tissues, including bone marrow (iliac crest, vertebral body, cancellous bone) [4,5], adipose tissue [6], skeletal muscle [7], dental pulp [8], periosteum [9], synovium [10], and gestational products. MSCs derived from human bone marrow or from human gestational products are described in Chapters 3–5. MSCs are typically identified and characterised in accordance with criteria proposed by the International Society of Cellular Therapy [11] as follows. Adherence to plastic; multipotent mesodermal differentiation potential *in vitro*; and the following CD (cluster of differentiation) marker profile: ≥95% $CD90^+$,

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$CD73^+$, $CD105^+$ and $\leq$95% $CD45^-$, $CD34^-$, $CD14/CD11b^-$, $CD79\alpha/CD19^-$, and $HLA\text{-}DR^-$. Although stromal cells from the tissues mentioned may all be termed MSCs, differences in phenotype, response to growth factors, and the molecular regulation of their lineage commitment suggests discrete differences between these cell populations. Since the *in vivo* characterization of these stromal cells is still lacking, our knowledge and understanding of these cell populations is limited principally to cells that have been expanded as monolayer cultures on tissue culture plastic. After over 40 years of MSC research it is now very clear that this method of culture induces wide-ranging phenotypic changes in a temporal manner. Thus, certain aspects of observed MSC behavior may not only be artefactual, but may also prevent us from understanding the true nature of these cells. As we will discuss later, this has significant implications not only for *in vitro* research but also for clinical transplantation.

From the few studies that have investigated the MSC niche in bone marrow, it is evident that MSCs are not simply one cell type, but a heterogeneous population, exhibiting discrete phenotypic differences, including ability to self-renew and degree of lineage commitment. This partly accounts for the range in nomenclature used when describing this cell population (mesenchymal stem cells, MSCs, mesenchymal progenitor cells, reticular cells). Moreover, there is also uncertainty as to whether pericytes – the vascular stromal support cells – may in fact be equivalent to MSCs, a subpopulation or their developmental precursor. For the purposes of this chapter, we refer to the population of plastic-adherent stromal cells which may be isolated from the mononuclear cell fraction of bone marrow or adipose tissue as MSCs or adipose stromal cells (ASCs) respectively.

In this chapter we describe the phenotype of MSCs isolated and cultured in monolayers and compare them with what is known of the MSC phenotype *in vivo*. We pay particular attention to the influence of isolation method and culture conditions on a range of phenotypic characteristics as measured *in vitro*. Finally, we discuss the implications of phenotypic changes upon both basic and clinical research. Bone marrow represents the most commonly used and traditional tissue source of MSCs. However, in the past decade there has been a significant increase in the body of adipose-derived MSC (ASC) research, due mainly to the greater ease and less invasiveness of collecting adipose tissue (by liposuction) compared with bone marrow aspiration. Thus, in this chapter we will focus primarily on MSCs derived from human bone marrow with reference to ASCs where appropriate. MSCs can also be isolated from human gestational organs, and these are discussed in Chapters 4 and 5.

## 7.2 Mesenchymal stromal cell isolation from bone marrow

In the absence of a universal standard isolation protocol, MSCs are isolated from bone marrow by different laboratories using different methods; these include plating whole bone marrow, red blood cell lysis, density centrifugation, and magnetic activated cell sorting (MACS)/fluorescence activated cell sorting (FACS). For MSC isolation, bone marrow is most frequently processed by density centrifugation, using Ficoll™, Lymphoprep™, or Percoll™ density media. This process separates the mononuclear cell (MNC) fraction from the other cellular and noncellular constituents of the marrow aspirate (e.g., red blood cells, plasma, and lipids). The resulting MNC fraction comprises a mixed population of cells, including T cells, B cells, monocytes, hematopoietic stromal cells, endothelial progenitor cells (EPCs) and MSCs. This mixed population of cells is seeded on to tissue culture plastic and is generally cultured for 3–5 days prior to the first media change. During this period, MSCs adhere to the tissue culture plastic and begin to form colonies. Hematopoietic, nonadherent cell populations are subsequently removed by serial media changes. It is also possible to isolate EPCs from the MNC fraction. However, their selection and expansion typically requires a basement membrane substrate such as fibronectin and the presence of specific growth factors [12]. A schematic for human bone marrow processing and MSC isolation procedures used in our laboratory is shown in Figure 7.1.

The density centrifugation process itself reduces the total yield of MNCs from bone marrow [13–15]. Plating whole bone marrow directly has been reported to increase CFU-F efficiency and to also result in a population of MSCs demonstrating longer telomere length compared with isolation protocols using either Ficoll or Percoll [14]. However, another group described reduced CFU-F efficiency by plating whole marrow compared with either density centrifugation or red blood cell lysis [16]. This same group demonstrated no difference in mesodermal differentiation potential or CD marker profile between cells isolated using either of the three techniques, although differences in cellular growth kinetics were less clear. Other laboratories use magnetic bead separation techniques, which deplete the polymorphonuclear cell population on the basis of granularity or expression of CD15. MNC recovery from both rat and human bone marrow was reported to be highest after MACS separation, followed by Percoll and then Ficoll density centrifugation [15]. Conversely, other studies directly comparing the efficiency of Ficoll with Lymphoprep [13] or with Percoll showed no significant difference in MNC yield [14]. Importantly, there is an increasing body of evidence to suggest that an MSC population is present in the initial, nonadherent MNC fraction [17–20]. This initially nonadherent MSC population is capable of forming CFU-Fs by serial plating. Importantly, the survival and subsequent expansion of these cells was shown to be critically dependent upon fibroblast growth factor-2 (FGF-2) signaling [20]. These MSCs demonstrated greater proliferation and tri-lineage differentiation potential *in vitro* and improved osteogenesis *in vivo* compared with a standard monolayer culture of MSCs. Together, these studies suggest the existence of heterogeneous MSC populations in the MNC fraction, and that those in the initially nonadherent fraction may represent a more naive stromal cell population. Owing to the apparent selection of an MSC population from the

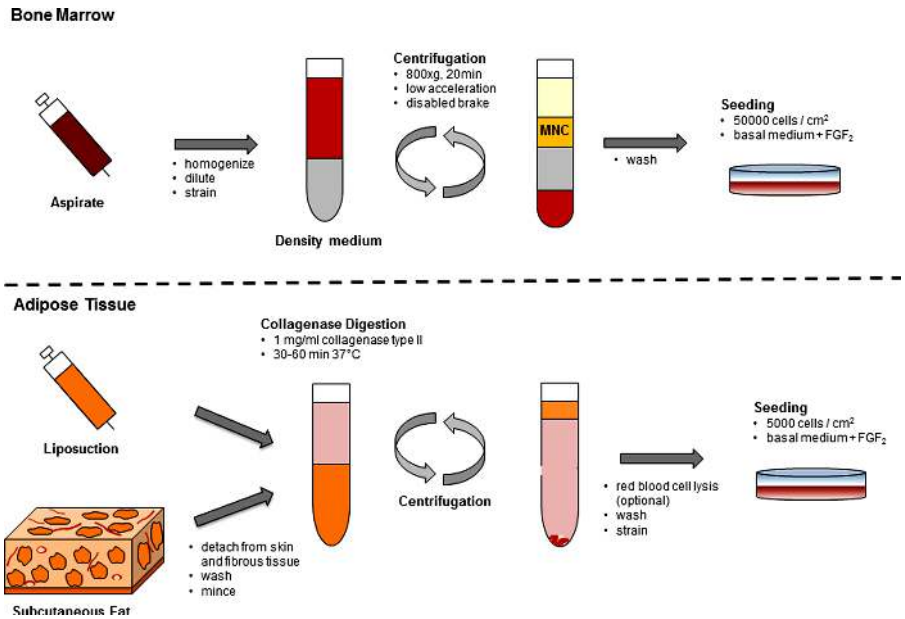



**Figure 7.1** Isolation of MSCs from bone marrow and adipose tissue. Schematic representation of standard experimental procedures for the isolation of mesenchymal stem cells isolated from bone marrow aspirate and adipose tissue (liposuction material/resection tissue). $FGF_2$: fibroblast growth factor-2.

original marrow aspirate, we propose that the term monolayer selection is adopted when discussing the isolation and culture of MSCs using the plastic adherence method [21]. MSCs are grown in basal medium such as Dulbecco's modified Eagle's medium (DMEM)/DMEM F12 or alpha minimum essential medium (MEM) with 10% fetal bovine serum (FBS) and cultured at 37 °C in 5% $CO_2$ and 20% $O_2$. One refinement of the isolation of MSCs from marrow is an initial purification step using a Stro-1 or similar antibody.

## 7.3 Mesenchymal stromal cell isolation from adipose tissue

Solid tissues such as adipose tissue, periosteum, and synovium are usually first dissected with scissors and scalpels prior to digestion with enzymes such as collagenase to break down the extracellular matrix prior to cell seeding and *ex vivo* expansion. ASCs are obtained from adipose tissue harvested by tissue resection or liposuction from thighs, flank, breast, or abdomen [22,23]. Adipose tissue is composed of two cell fractions: mature adipocytes and the stromal vascular fraction (SVF). The SVF is a heterogeneous cell mixture containing endothelial cells, hematopoietic cells, and an MSC population [24]. The frequency of stromal cells in the SVF is 1–10%, which is significantly higher than their frequency in bone marrow [23,25]. Donor age, harvest site, and isolation methods appear to affect yield and growth characteristics of the subsequently derived ASC population [22,23,26]. A schematic of the adipose tissue processing and cell isolation procedure used in our laboratory is shown in Figure 7.1. Tissue samples are first dissected to remove surrounding fibrous tissue and skin tissue, washed with phosphate-buffered saline (PBS) and then minced into small pieces. Cells are extracted from the minced tissue or liposuction sample by digestion with an equal volume of collagenase type II 1 mg/mL in cell culture medium with 3.5% bovine serum albumin (BSA) for 30–60 min at 37 °C. The fatty layer and adipocytes are removed by centrifugation and the cell pellet filtered through a 70 μm nylon mesh filter followed by additional washing with PBS. Afterwards, red blood cell lysis can be performed for samples containing large numbers of erythrocytes. Finally, a cell count is determined and cells seeded at a suitable density (approximately 3000/$cm^2$) in basal medium such as DMEM/DMEM F12 or alpha MEM with 10% FBS with or without FGF-2 and cultured at 37 °C in 5% $CO_2$ and 20% $O_2$.

As with bone marrow MSC isolation, seeding and expansion in a monolayer culture selects tissue culture plastic-adherent cells from the heterogeneous SVF, giving rise to a population of multipotent adipose-derived stromal cells (ASCs). The terminology ASC was first introduced by the International Fat Applied Technology Society (IFATS) referring to the multipotent plastic-adherent cell population. Prior to that, a variety of nomenclature has been used to describe this cell population, including "adipose-derived adult stromal cells," "adipose-derived stromal cells," "ASCs," "adipose mesenchymal stromal cells," "preadipocytes," and "processed lipoaspirate cells" [27].

## 7.4 Biological characteristics

### 7.4.1 Morphology and colony formation

When isolated on tissue culture plastic, MSCs adhere and proliferate, forming colonies. The heterogeneous nature of these cell populations is visibly evident at passage (p) 0 with morphologically distinct cell types present (Figure 7.2A and B). Small, spindle-shaped cell colonies are typically fast growing and more

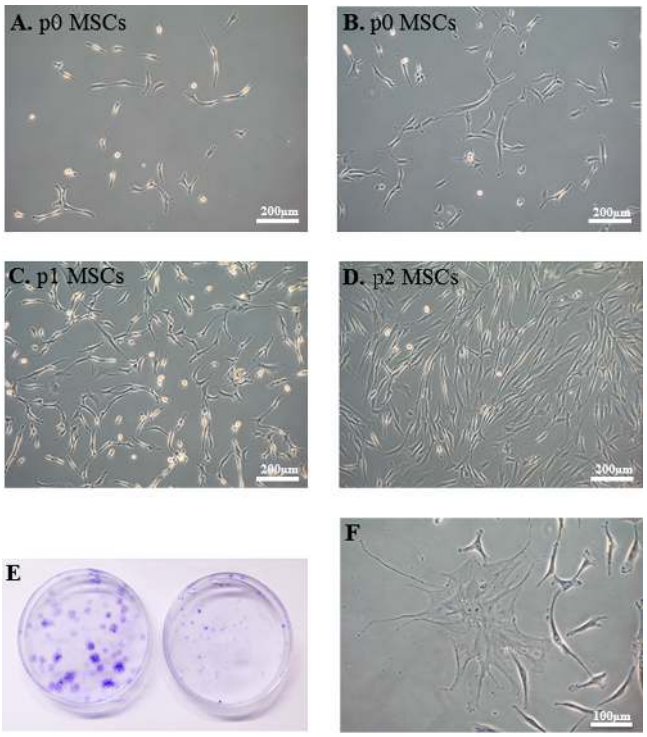



**Figure 7.2** Cellular morphology and colony-forming unit ability of MSCs cultured in monolayer. Phase-contrast images of human bone-marrow-derived mesenchymal stem cells at (A, B) p0, (C) p1, and (D) p2. (E) Crystal-violet-stained MSC colonies from a colony-forming unit (CFU) efficiency assay. (F) Visualization of stress fibers in an enlarged, senescent MSC.

abundant early on in the culture period. A population of larger cells is also present that appear to be less proliferative [28,29]. After subsequent passaging (p1) and expansion in monolayer culture, the cells become morphologically more homogeneous (Figure 7.2C and D).

The proportion of MNCs that attach and subsequently form MSC colonies may be described in terms of CFU efficiency. Colonies can be counted manually or by using automated techniques by first fixing the cells and then applying a suitable stain such as crystal violet (Figure 7.2E). CFU efficiency may be calculated using the following formula:

$$\text{CFU \% efficiency} = \frac{\text{number of colonies}}{\text{number of cells originally seeded}} \times 100$$

### 7.4.2 Growth kinetics

When calculating and describing MSC growth kinetics *in vitro*, passage number and/or population doublings may be referred to. However, since both cell size and the time taken to reach confluence varies significantly between donors, the passage number alone is not sufficient to provide accurate information as to growth rate. This may not only hinder data interpretation, but also prevents reliable comparison of MSC growth kinetics between laboratories. Population doublings may be calculated by using the following formula, which takes the initial cell number and final cell number during a single passage into consideration:

$$\log_{10}\left(\frac{\text{NF}}{\text{NO}}\right) \times 3.33$$

where NF is the final cell number and NO is the original cell number.

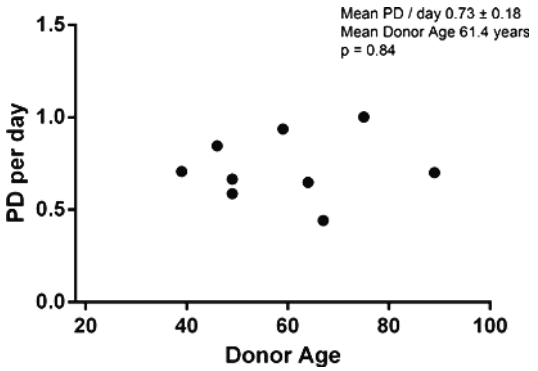



**Figure 7.3** Donor variation of MSC growth kinetics. Growth kinetics of bone-marrow-derived mesenchymal stromal cells harvested from vertebral body. Population doublings (PDs) recorded for MSCs between passage 1 and 2 show high donor variability and no correlation between growth rate and donor age. Pearson correlation coefficient was performed to test for correlation of PD and donor age.

Growth kinetics of MSCs are highly variable between different donors. Results from several studies suggest that older individuals demonstrate reduced initial CFU efficiency compared with younger cohorts [30,31]. However, donor characteristics such as age or gender cannot be used to reliably predict proliferation rate during extended culture periods. Figure 7.3 shows the population doubling time for nine human MSC donors, all from vertebral body bone marrow aspirates, indicating high variability but no correlation in terms of donor age. It is most likely that the variation in growth rate between MSC donors is attributed to sample variation in the original bone marrow aspirate. For example, when a bone marrow aspirate is taken, blood from the marrow sinusoids may also be aspirated, resulting in dilution of the bone marrow. Also, the proportion and number of more primitive and proliferative MSCs with a more "stromal-like" phenotype present in the aspirate is likely to be variable between separate aspirates and between different donors [32]. This has been clearly demonstrated by comparing the phenotype of MSC populations derived from two serial bone marrow aspirates from the same person [33].

With regard to ASCs, it has been proposed that population doubling time correlates with donor age, with a significantly lower proliferation rate of cells obtained from older donors [34]. As with bone-marrow-derived MSCs, it appears that the growth kinetics of ASCs are also dependent, in part, on the initial harvest method [22].

After an extended time in culture, MSCs will ultimately undergo replicative senescence [28,29,35]. The point at which a primary human MSC culture will undergo senescence is very variable between donors, but is typically seen between 10 and 38 population doublings [28,29,30,35]. Similarly, increased senescence has been reported after serial passaging of ASCs [34]. Stress fibers formed from actin-myosin cytoskeletal filaments are readily visualized by phase-contrast microscopy and are a good indicator of the replicative senescence of MSCs (Figure 7.2F). Senescence may be more reliably confirmed by staining cells for β-galactosidase, whereby 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) is cleaved, resulting in a blue–green substrate [36]. This lysosomal enzyme is present in most mammalian cells and detectable at pH 4. However, it is much more abundant in senescent cells, enabling it to be detected at the suboptimal pH of 6.

MSCs, as with most primary human cells, senesce during monolayer culture as a result of the Hayflick limit (after Leonard Hayflick, born 1928, an American anatomist), caused by telomere shortening [30,37]. DNA methylation and other epigenetic modifications may also contribute to the replicative senescence of MSCs [33,38]. In ASCs it has been shown that the frequency of chromosomal aberrations significantly increases after five passages [39]. With regard to long-term *in vitro* cultures, chromosomal stability should certainly be considered with respect to both the safety and efficacy of cell-based therapies.

### 7.4.3 *In vitro* multipotency

A universally accepted criterion defining *in vitro* cultured MSCs is their ability to differentiate along chondrogenic, osteogenic, and adipogenic lineages. For chondrogenesis, this is typically performed by culturing the cells as spherical pellets/micromass cultures and exposing them to a defined chondrogenic medium containing ascorbic acid, dexamethasone, insulin–transferrin–selenium, and TGF-β [40]. For osteogenic induction, MSCs can be cultured in monolayers or in three dimensions as pellets in osteogenic differentiation medium containing ascorbic acid, dexamethasone, and β-glycerophosphate. Adipogenic induction is achieved in monolayer culture by supplementation with 3-isobutyl-1-methyl xanthine, insulin, indomethacin, and dexamethasone. Although these *in vitro* assays demonstrate the quintessential multipotent MSC phenotype, there is currently no evidence to suggest that they are predictive of the ability or behavior of *in vitro* cultured MSCs to undergo lineage commitment and differentiation once implanted *in vivo*. As such, the relevance of these assays is a topic of much debate among researchers. The exception to this, however, is that the serial transplantation of MSC clones (CFU-Fs) generated from a single mesenchymal stem cell does result in both mesenchymal stem cell renewal and osteogenic differentiation in which the transplanted ossicle is populated by the recipient animal's hematopoietic system (see Chapter 32). Thus, the mesenchymal stem cell (as opposed to MSCs) is the stem cell of the skeleton.

After prolonged *in vitro* culture, MSCs appear to lose multipotency and display a propensity toward osteogenic differentiation [33,35,41]. Osteogenic lineage commitment appears to coincide with a reduction in proliferation and gene expression changes [37,41]. Other studies reported donor-dependent differential gene expression of integrins [42], extracellular matrix molecules, growth factors, and cytokines, including C–X–C motif chemokine 12 (CXCL12, also known as stromal-derived factor 1 (SDF-1)) [43] throughout the culture period.

### 7.4.4 Gene expression

Analysis of MSCs cultured in monolayers has demonstrated that gene expression changes occur directly after isolation and continue throughout culture. After extended culture, senescent MSCs downregulate genes associated with the cell cycle, DNA replication, and repair [41]. Their gene expression profile is also highly influenced by culture conditions and cell source [44,45]. The expression of specific stem cell markers, also referred to as "stemness" markers, has been studied in detail in embryonic stromal cells. Candidate markers for MSCs include the transcription factors OCT-4, NANOG, and SOX2. The expression of these transcription factors in MSCs is variable and likely dependent upon tissue source and culture conditions. MSCs that were shown to express OCT-4 and NANOG did not express SOX2 [46]. Other studies have failed to detect these markers in MSCs. This might be attributed to genotypic differences between the MSC populations isolated by different laboratories or to the detection methods used [47]. Interestingly, the latter study identified three genes – attenuated familial adenomatous polyposis (AFAP), protein tyrosine phosphatase receptor type F (PTPRF), and RAB3B, which encodes Ras-related protein Rab-3B – which were differentially regulated when MSCs were stimulated to undergo either osteogenic, adipogenic, or chondrogenic differentiation. Furthermore, the expression level of these genes was restored upon de-differentiation of the cells [47]. In order to identify MSC-specific genes, a comparative gene expression study comparing MSCs with other stromal cell populations revealed that colony-forming cells were enriched for *NOTCH3* (which encodes the neurogenic locus notch homolog protein 3), *ITGA11* (which encodes integrin α11), and *JAG1* (which encodes jagged 1 protein).

*NOTCH3*-positive cells displayed superior multipotency, while *ITGA11* expression indicated a more osteogenic progenitor phenotype [48]. Lineage commitment of MSCs is associated with gene expression changes, and differentially expressed genes have been identified during adipogenic, osteogenic, and chondrogenic differentiation. For a concise review, see reference [49] and Chapters 11–14. They will not be addressed in this chapter. However, while most of these typical genes are upregulated in the later stages of lineage commitment, recent work suggests that the ratio of the osteogenic gene runt-related transcription factor 2 (*RUNX2*) and the chondrogenic gene sex-determining region Y-box 9 (*SRY*, also known as *SOX9*) may be used to predict the fate of MSCs at much earlier stages [50].

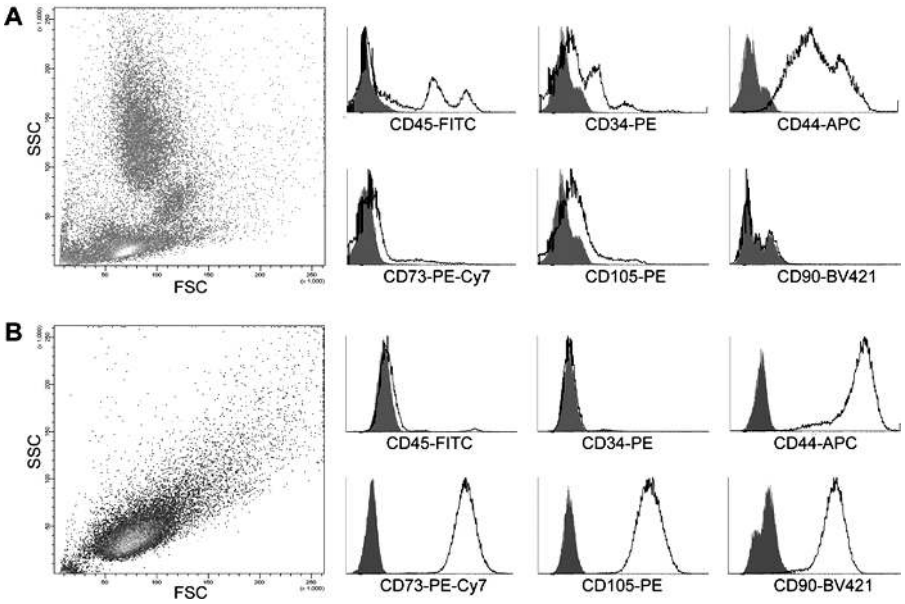



**Figure 7.4** Flow cytometry analysis of cell surface markers. (A) Flow cytometry dot plot depicting a representative profile of MNCs in respect of size (FSC: forward scatter) and granularity (SSC: site scatter) revealing that the MNC fraction consists of multiple cell populations expressing different levels of surface markers (filled dark curves indicate unstained controls). (B) Analysis of monolayer cultured MSCs upon first passage. The FSC–SSC plot reveals only one major cell population representing MSCs as selected by plastic adherence. In line with the current literature, monolayer cultured MSCs are negative for the hematopoietic markers CD45 and CD34 but express CD44, CD73, CD105, and CD90.

### 7.4.5 Cell surface marker profile

Human monolayer-expanded MSCs express various cell membrane-associated proteins that are used for identification and characterization purposes. The first of these markers to be recognized were CD73 [2,3] and CD105 [3,51]. Since there is no cell surface marker unique to MSCs, combinations of several cell surface markers are typically used to distinguish MSCs from other nucleated cell populations. In 2006 the International Society for Cellular Therapy recommended a panel of positive and negative cell surface markers to define MSC populations [11]. According to these guidelines, MSC populations were defined as being >95% positive for CD105, CD73, and CD90 and >95% negative for CD45, CD34, CD14, CD11b, CD79α or CD19, and HLA-DR. Note, however, that $CD34^+$ has been detected in tissue-resident MSCs but not on *ex vivo* expanded MSCs. A typical flow cytometry profile showing a heterogeneous population of bone-marrow-derived MNCs and the subsequently derived MSC population at passage 1 is shown in Figure 7.4.

CD105 is endoglin. CD73 is ecto-5′-nucleotidase. CD90 is Thy1. CD45 is the common leukocyte antigen. CD34 is an antigen present on hematopoietic stem and progenitor cells, EPCs, endothelial cells, pleural lymphatics vessels, mast cells, a subpopulation of dendritic cells in the interstitial tissues, around the adnexa of dermis of skin, and on tissue-resident MSCs. CD14 is a co-receptor for bacterial lipopolysaccharide. CD11b is an integrin family member that pairs with CD18 to form the complement receptor 3 heterodimer. CD11b is also a receptor for fibrinogen gamma chain, factor X, and intercellular adhesion molecule 1 (ICAM-1), also known as CD54. CD79α forms a complex with the B cell receptor (BCR) and generates a signal following recognition of antigen by the BCR. CD19 is a B cell surface marker. HLA-DR is a major histocompatibility complex class II cell surface receptor encoded by the human leukocyte antigen complex on chromosome 6 region 6p21.31.

Additional cell surface markers known to be expressed by MSC populations are CD44 [3,52], CD166 [53,54], CD106 [3,55], Stro-1 [56], CD146 [54,55], and CD271 [42]. CD44 is the receptor for hyaluronic acid and is also known as homing cell adhesion molecule. CD166 is also known as activated leukocyte cell adhesion molecule. CD106 is vascular cell adhesion molecule 1. Stro-1 (stromal precursor antigen-1) is a protein present on MSCs and on endothelium. CD146 is also known as melanoma cell adhesion molecule or cell surface glycoprotein MUC18, a mucin cell surface glycoprotein. CD271 is the low-affinity nerve growth factor receptor.

There are several important points to consider concerning the MSC markers described above, including their specificity, the transient nature of expression, and functional relevance. Other cell types of mesenchymal origin, including fibroblasts [57] and intervertebral disc cells [58], show a similar CD marker profile to that of MSCs. Moreover, we know that the cell surface marker expression profile of MSCs is affected by *in vitro* culture conditions.

Table 7.1 summarizes differences between the cell surface marker profiles of both human and murine MSCs in the bone marrow *in vivo*, freshly isolated cells, and in those that have been expanded *ex vivo* in monolayer culture on plastic. CD73 and CD105 appear to be expressed by the majority of freshly isolated MSCs with CFU-F capability, and are robustly maintained throughout culture [43,54]. Interestingly, it has been demonstrated that freshly isolated MSCs from both humans and mice do not express CD44, and that expression of this marker is acquired early on in culture [43]. Using DNA microarrays the same authors demonstrated that CD73 and the putative pericyte/

**Table 7.1** A comparison of cell surface marker expression profiles of human and murine *in vivo*, freshly isolated and monolayer-expanded mesenchymal stem or stromal cells

| Marker | *In vivo* | Freshly isolated | Post-monolayer expansion | Notes |
|---|---|---|---|---|
| **CD44** | ? | – [43] | + [43] | Mouse and human |
| | | | + [59] | Human |
| **CD73** | + [60] | | | Human, immunohistochemistry |
| | | + [54] | + [54] | Human, previously freeze-thawed |
| | | + [43] | + [43] | Mouse and human. ↑gene expression with time in culture |
| | | | + [61] | Human |
| **CD105** | | + [54] | + [54] | Human, previously freeze-thawed |
| | ? | + mouse/low human [43] | + | Human. ↑ gene expression with time in culture |
| | | | + [61] | Human |
| **CD90** | ? | ? | + [61] | Human |
| **CD166** | ? | ? | + [61] | Human |
| **CD271** | +/– [60] | – | | Human, niche location-dependent, immunohistochemistry |
| | | + [43] | | Human |
| | | + [62] | + [63] | Human |
| | + [63] | + [63] | – [61] | Human<br>Human, ↓ cell surface marker labeling with time in culture |
| **CD146** | +/– [60] | | | Human, niche location-dependent, immunohistochemistry |
| | | + [43] | + [54] | Human, ↑ gene expression in culture |
| | | + (variable) [54] | + [59]<br>+ [61] | Human, freeze-thawed<br>Human, ↓ with time in culture |
| | | | + [60] | Human, initially negative cells – acquired expression during culture |
| | | +/– [64]<br>+/– [63] | | Human, ↑ with time in normoxic culture, ↓ under hypoxia |
| **CD106** | ? | + (variable) [54] | + [54] | Human, previously freeze-thawed<br>Mouse and human |
| | | + [43] | + [59] | Human, ↓ cell surface marker labeling with time in culture |
| **GD2** | + [60] | | | Human, niche-dependent |
| | | + [65] | + [65] | Immunohistochemistry |
| | | | | Human |
| **Sca-1** | | + [43] | | Mouse |
| | + [66] | + [66] | ? | Mouse |
| | | + [67] | | Mouse |
| | + [67] | | | Mouse |

**Table 7.1** (*Continued*)

| Marker | *In vivo* | Freshly isolated | Post-monolayer expansion | Notes |
|---|---|---|---|---|
| **Stro-1** | | + [43] | | Human |
| | ? | | + [56] | Human |
| **Nestin** | | + [43] | ? | Mouse |
| | + [68] | + [68] | | Mouse |
| **PDGFr** | ? | + [43] | ? | Mouse |

Positive (+) and negative (–) cell surface antigen expression.
CD: cluster of differentiation; GD2: neural ganglioside 2; Sca-1: stem cell antigen-1; Stro-1: stromal derived factor-1; PDGFr: platelet-derived growth factor-1 receptor.
Note that cell surface antigen expression was ascertained by flow cytometry unless stated otherwise.

vascular niche MSC marker CD146 are upregulated during monolayer culture. Several studies indicate that CD271 and CD146 are expressed by subsets of freshly isolated MSCs and that cells initially negative for these markers acquire their expression during culture [42,43,54,59]. Conversely, another study reported that all CFU-F cells were present in either Lineage$^-$ CD45$^-$ CD271$^+$ CD146$^-$ or Lineage$^-$ CD45$^-$ CD271$^+$ CD146$^+$ sorted fractions [63]. Blocki *et al.* also demonstrated that initially CD146$^-$ MSCs acquire this marker during expansion in monolayer culture [64]. However, other groups have reported the loss of CD106 and CD146 expression during *in vitro* culture [59]. Discrepancies between these studies may be due to a range of factors including donor variation, different culture conditions, antibody clone and source, immunostaining protocol, FACS/magnetic bead sorting, and gating/analysis of flow cytometry data.

Significant changes in cell surface marker expression have also been observed in ASCs after monolayer selection and culture expansion. While ASCs in passage 2 or 3 expressed all the characteristic MSC markers listed by Dominici *et al.* [11], Gronthos *et al.* used flow cytometry and immunohistochemistry to also show that human adipose-tissue-derived stromal cells have a protein expression phenotype similar to that of human bone marrow stromal cells; expressed proteins included CD9, CD10, CD13, CD29, CD34, CD44, CD49d, CD49e, CD54, CD55, CD59, CD105, CD106, CD146, and CD166. Unlike human bone-marrow-derived stromal cells, they did not detect the Stro-1 antigen on human adipose-tissue-derived stromal cells [69]. CD9 is a member of the tetraspanin family of proteins, which are thought to act as scaffolding proteins, anchoring multiple proteins to one area of the cell membrane. CD10 is neprilysin, also known as membrane metallo-endopeptidase (MME), neutral endopeptidase (NEP), and common acute lymphoblastic leukemia antigen (CALLA). Neprilysin is a zinc-dependent metalloprotease that cleaves peptides at the amino side of hydrophobic residues and inactivates several peptide hormones, including glucagon, enkephalins, substance P, neurotensin, oxytocin, and bradykinin. CD13 is alanine aminopeptidase, an enzyme that is used as a biomarker to detect damage to the kidneys, and that may be used to help diagnose certain kidney disorders. CD29 is integrin β1. CD49d is an integrin alpha subunit and is also known as very late antigen-4 (VLA-4). It makes up half of the α4β1 lymphocyte homing receptor. CD49e is integrin α5 and is also known as VLA-5. CD54 is intercellular adhesion molecule 1 (ICAM-1). CD55 is complement decay-accelerating factor (DAF), a protein that regulates the complement system on the cell surface. CD59 is a glycoprotein also known as MAC-inhibitory protein (MAC-IP), membrane inhibitor of reactive lysis (MIRL), or protectin. CD59 attaches to host cells via a glycophosphatidylinositol anchor. When complement activation leads to deposition of C5b678 on host cells, CD59 can prevent C9 from polymerizing and forming the complement membrane attack complex.

Some markers are altered within the stromal cell population present in freshly isolated stromal vascular fractions. Although no specific marker profile identifying colony-forming cells has been found, most of the literature suggests that ASCs are contained in the CD34$^+$ cell fraction. Interestingly, it has been suggested that typical markers of monolayer cultured MSCs/ASCs such as CD105 and CD73 were absent or low on freshly isolated ASCs but were dramatically increased after plating on plastic [70,71].

The cell surface marker expression profiles of MSC populations within the bone marrow milieu have also been investigated immunohistochemically [60,63]. CD73 is widely expressed by morphologically heterogeneous MSC populations in stromal, endosteal and perivascular locations [60], whereas CD271 is expressed by cells within the stroma and at the endosteal surface. In accordance with previous work, CD146$^+$ cells were exclusively localized immunohistochemically in perivascular regions. Rasini *et al.* also reported expression of neural ganglioside GD2 by endosteal and perivascular MSCs, supporting a previous study identifying this neural protein as a marker of MSCs before *ex vivo* expansion [60]. Conversely, Tormin *et al.* did not observe any immunostaining of GD2 by MSCs in bone marrow. Notably,

OCT4, NANOG, and stage-specific embryonic antigen-4 (SSEA-4) were immunolocalized to MSCs at endosteal and stromal locations but not in perivascular structures, suggesting that phenotypically different MSC populations reside in anatomically distinct niches within the marrow [63] and see Chapter 32.

Taken together, it appears that the cell surface marker profile of MSCs *in vitro* is subject to change as a result of artificial culture conditions and is not necessarily representative of their expression profile *in vivo*. Importantly, differential marker expression has not been correlated to changes in cell characteristics such as growth kinetics or plasticity, which brings their functional significance into question. In practical terms, cell surface marker profiling becomes important when dealing with freshly isolated, heterogeneous cell populations and in co-culture studies with other cell types. Further investigation of the functional relevance of differentially expressed markers by specific MSC populations within the bone marrow may help further understanding of the biological significance of stromal, endosteal, and perivascular niches and their constitutive stromal cell populations (see Chapter 32).

### 7.4.6 Secretory profile

The secretory profile of tissue-specific MSCs within their *in vivo* environments is largely unknown. Conversely, extensive literature exists regarding the composition and effects of the secretome from MSCs cultured *in vitro*. Bone-marrow-derived mesenchymal stromal cells secrete a multitude of extracellular matrix molecules, growth factors, cytokines and chemokines when cultured in monolayers [72–74]; and see Chapters 18 and 19. The presence and level of such molecules have significant implications with regard to autocrine signaling when the cells are expanded in monolayer and in a paracrine context when exposed to other cell types in co-culture systems. Moreover, it is widely believed that the therapeutic effects observed following MSC transplantation may largely be attributed to the release of trophic factors [75]. MSC-secreted factors may be categorized in terms of physiological function: mitogenesis, cell survival/anti-apoptosis, chemotaxis, immunomodulation, angiogenesis, and neurogenesis. A detailed review of the MSC secretome has been published by Da Silva Meirelles *et al.* [75] and Hsiao *et al.* [74]; and see Chapters 18 and 19. Similarly, ASCs have been shown to release soluble factors modulating angiogenesis, inflammation, and apoptotic signaling [76].

MSCs produce many anti-inflammatory factors, including hepatocyte growth factor (HGF) [72,77], cyclooxygenase 1 and 2, interleukin-10 (IL-10), prostaglandin $E_2$ [78], interleukin-1 receptor antagonist (IL-1Ra) [79], and tumor necrosis factor α-induced protein 6 (TSG-6) [80]. When cultured in monolayers MSCs produce pro-angiogenic factors – including vascular endothelial growth factor (VEGF), FGF-2, placental growth factor, angiopoeitin-1, platelet-derived growth factor (PDGF), matrix metalloproteinase-9, monocyte chemoattractant protein-1 (MCP-1) [72] – and the neurotrophic factors brain-derived neurotrophic factor [81,82], nerve growth factor [81], neuroregulin-1 (NRG1-β1) [83], glial-derived neurotrophic factor (GDNF) [84], artemin, and persephin [85]. Artemin is a neurotrophin in the glial cell line-derived neurotrophic factor (GDNF) family of ligands, which are a group of ligands within the TGF-β superfamily of signaling molecules. GDNFs are unique in having neurotrophic properties and have potential use for gene therapy in neurodegenerative diseases. Persephin is also a neurotrophic factor in the glial cell line-derived neurotrophic factor family.

Neutralizing antibody experiments suggest that the mitogenic effects of the MSC secretome are not attributable to the presence of one particular growth factor, but are the result of the combinatorial effects of many factors [72].

It is important to consider that the secretory profile of MSCs may be modulated by different *in vitro* culture techniques. It is known, for example, that transition from two-dimensional to three-dimensional culture results in an increase in the production of pro-angiogenic factors [86]. Differentiation status has also been shown to affect the secretory profile of MSCs. Specifically, when differentiated osteogenically or chondrogenically, human and equine MSCs reduce production of angiogenic and neurogenic factors and demonstrate anti-angiogenic effects *in vitro* [85,87]. The secretory profile of MSCs also appears sensitive to changes in oxygen tension. When cultured under hypoxic conditions (<5% $O_2$) cells increase production of SDF-1, angiogepoietin-1, and VEGF via hypoxia-inducible factor-1 (HIF-1) [88,89]. A detailed review on the effects of hypoxic preconditioning on the secretory profile of MSCs has been published by Hsiao *et al.* [74] and is discussed later in this chapter.

Of note, the time-related characteristics of the secretome by MSCs expanded in monolayer culture have not yet been reported and represent a significant gap in our knowledge. Moreover, we do not know how the secretome of *in vitro* cultured cells compares with that of unmanipulated MSC populations *in vivo* or with that of transplanted MSCs previously cultured *in vitro*. This information will be crucial for understanding the physiological function and realizing the full therapeutic potential of these cells.

## 7.5 Influences on tissue culture parameters

### 7.5.1 Seeding density

In the absence of standardized culture protocols, researchers culture MSCs at varying seeding densities, often between 2000 and 5000 cells/$cm^2$. Several studies suggest that lower seeding densities increase the rate of cellular proliferation by reducing contact inhibition [42,52,90,91]. Reducing seeding density has been reported not to affect the expression of CD markers or trilineage mesodermal differentiation capability *in vitro* [52,91]. It is important not to allow MSC cultures to become overconfluent during monolayer expansion since, besides contact inhibition

and nutritional restrictions which induce growth arrest and ultimately lead to cell death, this may also induce irreversible phenotypic changes and prime the culture to undergo osteogenic differentiation as a default pathway [35,38,41]. Thus, it is common practice to passage MSCs when the culture reaches approximately 80% confluency.

### 7.5.2 Culture medium and supplementation

A basal medium such as DMEM/DMEM F12 or alpha MEM containing 10% FBS is typically used to culture MSCs *in vitro*. The composition of growth media may affect MSCs at the genomic, transcriptomic, and proteomic levels. For example, in a study comparing the effects of different culture media on monolayer-expanded ASCs, cell morphology, growth kinetics, immunophenotype, and expression of stemness-related transcription factors were all significantly modulated [92]. FBS is subject to batch variation, which may also greatly affect aspects of MSC behavior, such as growth rate and multipotency. Therefore, serum testing should be performed in order to maintain consistent growth conditions. Alternatively, commercially available MSC-qualified batch-tested sera are available at a considerable cost to the user.

### 7.5.3 Growth factors

Supplementation of culture media with specific growth factors and cytokines influences the genotype and phenotype of MSCs. FGF-2 is a known mitogen of MSCs, also functioning to maintain multipotency and to promote subsequent chondrogenic differentiation *in vitro* [20,93–95]. There is evidence to suggest that FGF-2 supplementation may reduce CD146 expression in primary cells and cell lines [96,97]. However, as discussed previously in this chapter, the functional significance of this and other markers has yet to be elucidated. Concentrations between 0 and 10 ng/mL of FGF-2 are reported in the literature, adding another compounding factor when attempting to compare studies.

### 7.5.4 Xeno-free media

Although FBS is the most widely used medium supplementation for the *in vitro* expansion of MSCs, concerns arise when considering the clinical application of cells. Transplantation of FBS-cultured MSCs may cause immune reactions [98] and may be contaminated with bacteria, viruses, or prions [99]. For these reasons there has been a drive to establish FBS-free MSC culture methods by using autologous serum, platelet lysate (PL), platelet-rich plasma (PRP), or serum/plasma-free growth factor supplementation. A detailed review of this area was been published by Tonti and Mannello in 2008 [100]. It has also been reported that autologous serum maintains genomic stability whilst promoting the proliferation of primary MSCs [101]. Of note, the use of fetal calf serum (FCS) for the production of MSCs to be used in clinical trials in Australia is allowed by the Australian regulatory authority, the Therapeutic Goods Agency, as long as the FCS is sourced from a herd in a country that has never had an outbreak of bovine spongiform encephalopathy (mad cow disease). This is currently the case for Australia and New Zealand.

### 7.5.5 Platelet-derived supplements

PDGFs have been employed as an alternative to serum supplementation. Platelet-derived medium supplementation can be prepared from donor-matched blood and can be thus considered as xeno-free (i.e., free from any contamination by components of nonhuman species), with the autologous medium circumventing safety concerns associated with the use of FBS.

Although platelet products have been used for several years now, no consensus with regard to preparation methods and terminology exists. PL or platelet-released growth factors (PRGFs) generally refer to a concentrate of growth factors obtained after platelet activation in either the presence or absence of platelet-poor plasma. Commonly used activation methods include repetitive freeze–thaw cycles and sonication. PRP refers to a concentrate of platelets that may or may not be activated by the addition of thrombin and/or calcium chloride ($CaCl_2$), resulting in growth factor release and fibrin clot formation. Platelet preparations contain different growth factors, including FGF-2, TGF-β, and PDGF-BB, with inhibition of these factors resulting in a significant reduction in MSC proliferation in PL-medium [102]. In chemical terms, PDGF is a dimeric glycoprotein composed of two A (−AA) or two B (−BB) chains or a combination of the two (−AB).

Besides this there is also evidence that microRNAs and exosomes represent functional components of platelet products [99]. The actual growth factor content in platelet preparations is highly dependent upon the donor as well as on the preparation and activation method, which altogether will affect the phenotype and behavior of MSC cultures. For cell culture purposes, PL is favored over PRP preparations since these are rich in potentially disturbing platelet debris and have the risk of spontaneous aggregation and fibrin clot formation, although it may be possible to prevent the latter by the addition of heparin. Furthermore, in cultures of ASCs it has been shown that certain growth factors influencing cell proliferation are expressed at greater levels in PL compared with activated PRP [103].

Both PL/PRGF and PRP supplementation have been reported to increase MSC CFU-F efficiency and proliferation compared with both FBS and autologous serum [104–110]. An increase in proliferation rate has also been reported in low-density MSC cultures supplemented with PL under good manufacturing practice-compliant conditions [102]. Similarly, enhanced proliferation of ASCs was shown with PL supplementation [106,107]. Most studies suggest that this occurs without affecting CD marker profiles.

More importantly, functional parameters including gene expression, plasticity, and the MSC secretome are influenced by PL/PRP supplementation. For example, PRP increases the expression of genes associated with osteogenic and chondrogenic lineage commitment – *RUNX2*, *SOX9*, and *AGGRECAN* [106]. On the other hand, several studies using PL showed that MSCs

preserve their multipotency [108–110,113]. These conflicting outcomes are, again, likely attributed to variability in preparation procedures as well as to donor variability. This is supported by a study comparing the effects of PL donors of different ages [114]. In this particular study, a higher proliferation rate and enhanced osteogenic differentiation with PL from younger donors (<35 years of age) compared with PL from older donors (>45 years of age) was observed. Interestingly, these effects could not be correlated to levels of growth factors, including PDGF-AB, TGF-β1, FGF, IGF-1, or hormones, including estradiol, parathormone, leptin, or vitamin D3. The secretome of MSCs is also influenced by PRP or PL supplementation [107,110]. The secretion of IL-6 and IL-8 was shown to be increased by MSCs cultured with PL compared with FBS [110]. Additionally, PL supplementation is also associated with changes in MSC gene expression [109,110].

### 7.5.6 Serum-free media

The use of serum-free culture media for MSC expansion is reported in the literature and is commercially available at considerable cost. Serum-free cultures contain a number of different recombinant growth factors and generally involve a coating of cell culture plates to facilitate cell attachment. The main advantage of serum-free media is that they have a defined composition (although this is often proprietary) compared with serum or PL/PRP-containing media. While both FBS and PL/PRP exhibit batch and donor variation, serum-free media consist of an unchanging, defined mixture of growth factors and proteins.

A combination of PDGF-BB, FGF-2, and TGF-β1 has been reported to support MSC proliferation whilst maintaining multipotency [115]. Furthermore, it has been shown that proliferation rate, multipotency, and surface marker expression of MSCs cultured in commercially available serum-free supplements were generally comparable to MSCs cultured with FBS [116,117]. Conversely, serum-free cultures of MSCs were associated with greater levels of cell senescence at early passages, changes in the surface marker profile, and differentiation capacity [118]. Interestingly, these deleterious effects of serum-free culture media were not evident on ASC cultures. Besides the uncertainty over phenotypic stability, the application of serum-free media for the initial steps of MSC isolation and expansion might impair cell adherence to tissue culture plastic and CFU-F efficiency [99].

### 7.5.7 Hypoxia

Most laboratories culture MSCs under atmospheric oxygen tension (21% $O_2$). This, however, does not reflect the *in vivo* situation, where stromal cells reside in their niche with generally much lower oxygen tensions ranging between 1 and 5%. Accordingly, studies addressing MSC culture under low oxygen tension typically use oxygen levels within such physiological ranges. It is noteworthy that different oxygen tensions, together with different culture methods and conditions (including media composition, supplementation, seeding densities, and donor variation), are likely to influence experimental outcomes and thus hinder the comparability of results between studies. Nonetheless, the culture of MSCs under hypoxic conditions has been shown to influence many aspects of cell phenotype, such as growth kinetics, migratory capacity, gene expression, and the secretome.

When comparing MSC isolation and expansion under either hypoxic or normoxic conditions, a decrease in initial CFU-F efficiency under hypoxia was observed. However cells were more proliferative and outgrew normoxic cultured MSCs at passage 2 [119]. The majority of published studies have evaluated the effect of hypoxia on MSCs already selected by plastic adhesion. During monolayer expansion, it appears that low oxygen tension leads to increased cell proliferation and clonogenic potential, whilst cell morphology and surface marker expression are not significantly influenced [120–123]. These conclusions are highly dependent upon the exact culture parameters. This was particularly evident in a study comparing the colony-forming capability of MSCs cultured in different oxygen tensions, demonstrating a significantly different outcome when cells were cultured in either 2% or 5% $O_2$ [124].

The effect of hypoxic culture conditions on the differentiation potential of MSCs is not fully understood. On the one hand, it has been reported that multipotency is maintained under hypoxia [119,120]. In line with this, upregulation of the stemness marker genes *SOX2*, *OCT4*, and *NANOG* was reported when ASCs were incubated at 2% $O_2$ [122]. On the other hand, several studies have suggested that hypoxic conditions do indeed affect the differentiation potential of MSCs and ASCs [121–127]. These studies are thus conflicting.

In addition to culturing MSCs in hypoxic conditions during the monolayer expansion phase, hypoxic preconditioning prior to transplantation has also been investigated and proven beneficial [123,128]. Specifically, the upregulation of hypoxia-inducible factor 1α and its downstream targets promotes cell survival after transplantation *in vivo*. Moreover, it has been reported that hypoxia induced the expression of CMET via AKT signaling. This in turn supported cell migration and recruitment by HGF *in vivo* [123]. (CMET, a receptor tyrosine kinase, is also called MET and hepatocyte growth factor receptor; AKT, also known as protein kinase B, is a serine/threonine-specific protein kinase that plays a key role in multiple cellular processes such as glucose metabolism, apoptosis, cell proliferation, transcription, and cell migration. The "Ak" in Akt was a temporary classification name for a mouse bred and maintained that developed spontaneous thymic lymphomas. The "t" stands for thymoma.)

## 7.6 Implications for basic and clinical research

### 7.6.1 Trial disparity

Methods of bone marrow preparation and MSC isolation and *in vitro* expansion vary significantly between laboratories. Thus, the resulting MNC population and the *in vitro* phenotype

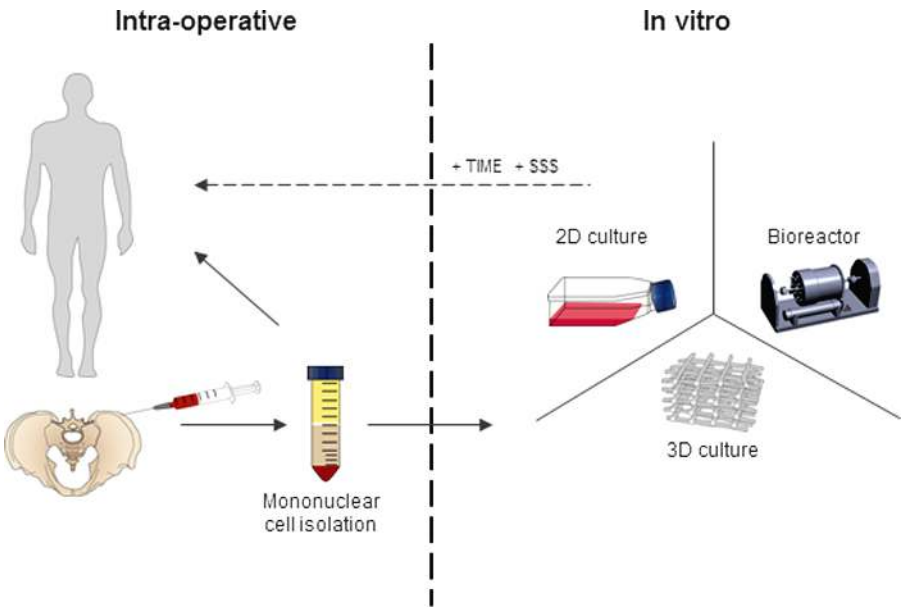



**Figure 7.5** Intraoperative versus *in vitro* approaches to mesenchymal stem cell therapy and tissue engineering.

acquired by MSCs during monolayer culture is also likely to vary between research groups. This is evident from observed discrepancies between *in vitro* and *in vivo* studies in published literature. Importantly, it also appears that seemingly minor differences in isolation and culture methodology may affect the clinical performance of both MNCs and MSCs, resulting in discrepancies in results between trials using these cell populations. Indeed, this was shown by a group who compared two very similar, randomized, placebo-based controlled trials in which MNCs were transplanted after myocardial infarction [129]. In this trial, known as the REPAIR-AMI (The Reinfusion of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial, improved left ventricular ejection fraction was reported following MNC transplantation [130], whereas no significant improvement in clinical outcome compared with placebo was reported in the ASTAMI (Autologous Stem cell Transplantation in Acute Myocardial Infarction) trial [131].

By replicating the isolation methods of both trials, Seeger's group demonstrated that the protocol used by researchers of the REPAIR-AMI trial yielded greater numbers of MNCs with enhanced CFU-F efficiency, improved therapeutic effect in a mouse model of hindlimb ischemia, and that MNC-derived MSCs were more responsive to SDF-1 *in vitro*. These differences appeared to be attributable to differences in culture media, centrifugation speeds used, wash steps and cell storage protocol. Additional variables, including the degree of red blood cell contamination and abundance of apoptotic cells have also been correlated with reduced clinical efficacy of transplanted MNCs [132,133].

### 7.6.2 Alternative culture systems

It is clear that a plethora of phenotypic changes are experienced by MSCs as a result of monolayer culture. Thus, the behavior of MSCs *in vitro* may largely be artefactual and likely not representative of their behavior *in vivo*. This has significant implications for basic researchers aiming to investigate the behavior and function of resident MSCs within bone marrow and adipose and other tissues. Modulation of MSC biology caused by monolayer expansion may ultimately compromise the therapeutic value of the cells. For example, several groups have reported improved multipotency and increased trophic factor release in MSC spheroid cultures generated from MSCs initially cultured in monolayers [134].

From both clinical and economic perspectives, the expansion of MSCs *in vitro* is a lengthy and costly process. Some investigators are now focusing on developing intraoperative therapies in which the MNC cell fraction is isolated and implanted during a one-step procedure and taking advantage of the capacity of MSCs to home to damaged tissues (Figure 7.5). However, the lack of knowledge we possess on the biology of tissue-resident MSCs is impeding the development of both such intraoperative MNC therapies and targeted homing strategies. For example, processes developed for monolayer-expanded MSCs may simply not be effective on a tissue-resident unmanipulated MSC population – either in *ex vivo* or *in vivo* systems. Thus, there is a need to develop alternative culture systems that avoid the use of tissue culture plastic, thereby maintaining MSCs in a more native state.

As an alternative to tissue culture plastic, MSCs may be cultured and expanded using microcarriers within stirred bioreactors [135–138]. These systems may also be used to generate clinical-grade cultures for human transplantation [139]. Three-dimensional culture systems, although not necessarily able to meet the demands of clinical scale-up, offer the opportunity to co-culture previously unmanipulated MSCs with other niche cells, thus better mimicking their *in vivo* environment. Several groups have demonstrated that mixed populations of MNCs,

including CFU-F-forming MSCs can be maintained *in vitro* either by using hydroxyapatite scaffolds under perfusion [140–142] or by culture within type I collagen hydrogels [143]. Refinement of culture systems such as these may provide us with the means to maintain MSC phenotype *in vitro* and therefore allow us to better study and understand their function *in vivo*.

## 7.7 Conclusions and future directions

Monolayer expansion on tissue culture plastic is the most widely used method for growing MSCs in *in vitro* culture. Selection and culture of these cells on plastic results in wide-ranging phenotypic changes that prevent us from understanding the true biology of these cells in their native resident *in vivo* state. Compounding this, there is great variation between research groups with regard to isolation and culture parameters. Although a significant challenge, the standardization, isolation, and expansion in monolayer culture would bring a greater degree of uniformity within the field. This would not only allow more reliable comparisons to be made between *in vitro* studies, but could also improve the consistency in clinical outcome observed after MSC transplantation. Furthermore, improved culture systems that maintain phenotypic stability and allow us to study these cells in more physiologically relevant environments will improve our understanding of the function of these cells *in vivo* and assist in the development of clinically efficacious and more economically viable MSC therapies.

## References


1 Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 1968;**6**(2):230–247.

2 Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**(4):393–403.

3 Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411): 143–147.

4 Noth U, Osyczka AM, Tuli R, *et al.* Multilineage mesenchymal differentiation potential of human trabecular bone-derived cells. *J Orthop Res* 2002;**20**(5):1060–1069.

5 Sakaguchi Y, Sekiya I, Yagishita K, *et al.* Suspended cells from trabecular bone by collagenase digestion become virtually identical to mesenchymal stromal cells obtained from marrow aspirates. *Blood* 2004;**104**(9):2728–2735.

6 Zuk PA, Zhu M, Ashjian P, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**(12):4279–4295.

7 Mastrogiacomo M, Derubeis AR, Cancedda R. Bone and cartilage formation by skeletal muscle derived cells. *J Cell Physiol* 2005; **204**(2):594–603.

8 Huang GT, Gronthos S, Shi S. Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine. *J Dent Res* 2009;**88**(9):792–806.

9 Nakahara H, Dennis JE, Bruder SP, *et al. In vitro* differentiation of bone and hypertrophic cartilage from periosteal-derived cells. *Exp Cell Res* 1991;**195**(2):492–503.

10 De Bari C, Dell'Accio F, Tylzanowski P, Luyten FP. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001;**44**(8):1928–1942.

11 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

12 Masuda H, Asahara T. Post-natal endothelial progenitor cells for neovascularization in tissue regeneration. *Cardiovasc Res* 2003;**58** (2):390–398.

13 Van Beem RT, Hirsch A, Lommerse IM, *et al.* Recovery and functional activity of mononuclear bone marrow and peripheral blood cells after different cell isolation protocols used in clinical trials for cell therapy after acute myocardial infarction. *EuroIntervention* 2008;**4**(1):133–138.

14 Mareschi K, Rustichelli D, Calabrese R, *et al.* Multipotent mesenchymal stromal cell expansion by plating whole bone marrow at a low cellular density: a more advantageous method for clinical use. *Stem Cells Int* 2012; **2012**: article ID 920581. doi: 10.1155/2012/920581.

15 Posel C, Moller K, Frohlich W, *et al.* Density gradient centrifugation compromises bone marrow mononuclear cell yield. *PloS One* 2012;**7**(12):e50293.

16 Horn P, Bork S, Wagner W. Standardized isolation of human mesenchymal stromal cells with red blood cell lysis. *Methods Mol Biol* 2011;**698**:23–35.

17 Wan C, He Q, McCaigue M, *et al.* Nonadherent cell population of human marrow culture is a complementary source of mesenchymal stromal cells (MSCs). *J Orthop Res* 2006;**24**(1):21–28.

18 Zhang ZL, Tong J, Lu RN, *et al.* Therapeutic potential of non-adherent BM-derived mesenchymal stem cells in tissue regeneration. *Bone Marrow Transplant* 2009;**43**(1):69–81.

19 Akiyama K, You YO, Yamaza T, *et al.* Characterization of bone marrow derived mesenchymal stromal cells in suspension. *Stem Cell Res Ther* 2012;**3**(5):40. doi: 10.1186/scrt131.

20 Di Maggio N, Mehrkens A, Papadimitropoulos A, *et al.* Fibroblast growth factor-2 maintains a niche-dependent population of self-renewing highly potent non-adherent mesenchymal progenitors through FGFR2c. *Stem Cells* 2012;**30**(7):1455–1464.

21 Bara JJ, Richards RG, Alini M, Stoddart MJ. Concise review: bone marrow-derived mesenchymal stromal cells change phenotype following *in vitro* culture: implications for basic research and the clinic. *Stem Cells* 2014;**32**(7):1713–1723.

22 Oedayrajsingh-Varma MJ, van Ham SM, Knippenberg M, *et al.* Adipose tissue-derived mesenchymal stromal cell yield and growth characteristics are affected by the tissue-harvesting procedure. *Cytotherapy* 2006;**8**:166–177.

23 Jurgens WJ, Oedayrajsingh-Varma M, Helder M, *et al.* Effect of tissue-harvesting site on yield of stem cells derived from adipose tissue: implications for cell-based therapies. *Cell Tissue Res* 2008;**332**:415–426.

24 Prunet-Marcassus B, Cousin B, Caton D, *et al.* From heterogeneity to plasticity in adipose tissues: site-specific differences. *Exp Cell Res* 2006;**312**:727–736.

25 Aust L, Devlin B, Foster SJ, *et al.* Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy* 2004;**6**:7–14.

26 Engels P, Tremp M, Kingham P, *et al.* Harvest site influences the growth properties of adipose derived stem cells. *Cytotechnology* 2013;**65**:437–445.

27 Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007;**100**:1249–1260.

28 Digirolamo CM, Stokes D, Colter D, *et al.* Propagation and senescence of human marrow stromal cells in culture: a simple colony-forming assay identifies samples with the greatest potential to propagate and differentiate. *Br J Haem* 1999;**107**(2):275–281.

29 Muraglia A, Cancedda R, Quarto R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* 2000;**113**(Pt 7):1161–1166.

30 Baxter MA, Wynn RF, Jowitt SN, *et al.* Study of telomere length reveals rapid aging of human marrow stromal cells following *in vitro* expansion. *Stem Cells* 2004;**22**(5):675–682.

31 Dexheimer V, Mueller S, Braatz F, Richter W. Reduced reactivation from dormancy but maintained lineage choice of human mesenchymal stem cells with donor age. *PloS One* 2011;**6**(8):e22980.

32 Fennema EM, Renard AJ, Leusink A, *et al.* The effect of bone marrow aspiration strategy on the yield and quality of human mesenchymal stem cells. *Acta Orthop* 2009;**80**(5):618–621.

33 Bork S, Pfister S, Witt H, *et al.* DNA methylation pattern changes upon long-term culture and aging of human mesenchymal stromal cells. *Aging Cell* 2010;**9**(1):54–63.

34 Gruber HE, Somayaji S, Riley F, *et al.* Human adipose-derived mesenchymal stromal cells: serial passaging, doubling time and cell senescence. *Biotech Histochem* 2012;**87**:303–311.

35 Bruder SP, Jaiswal N, Haynesworth SE. Growth kinetics, self-renewal, and the osteogenic potential of purified human mesenchymal stem cells during extensive subcultivation and following cryopreservation. *J Cell Biochem* 1997;**64**(2):278–294.

36 Dimri GP, Lee X, Basile G, *et al.* A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc Natl Acad Sci U S A* 1995;**92**(20):9363–9367.

37 Redaelli S, Bentivegna A, Foudah D, *et al.* From cytogenomic to epigenomic profiles: monitoring the biologic behavior of *in vitro* cultured human bone marrow mesenchymal stem cells. *Stem Cell Res Ther* 2012;**3**(6):47. doi: 10.1186/scrt138.

38 Banfi A, Bianchi G, Notaro R, *et al.* Replicative aging and gene expression in long-term cultures of human bone marrow stromal cells. *Tissue Eng* 2002;**8**(6):901–910.

39 Froelich K, Mickler J, Steusloff G, *et al.* Chromosomal aberrations and deoxyribonucleic acid single-strand breaks in adipose-derived stem cells during long-term expansion *in vitro*. *Cytotherapy* 2013;**15**:767–781.

40 Johnstone B, Hering TM, Caplan AI, *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**(1):265–272.

41 Wagner W, Horn P, Castoldi M, *et al.* Replicative senescence of mesenchymal stem cells: a continuous and organized process. *PloS One* 2008;**3**(5):e2213.

42 Fekete N, Rojewski MT, Furst D, *et al.* GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. *PloS One* 2012;**7**(8):e43255.

43 Qian H, Le Blanc K, Sigvardsson M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J Biol Chem* 2012;**287**(31):25795–25807.

44 Wagner W, Wein F, Seckinger A, *et al.* Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* 2005;**33**:1402–1416.

45 Torensma R, Prins H, Schrama E, *et al.* The impact of cell source, culture methodology, culture location, and individual donors on gene expression profiles of bone marrow-derived and adipose-derived stromal cells. *Stem Cell Dev* 2013;**22**:1086–1096.

46 Riekstina U, Cakstina I, Parfejevs V, *et al.* Embryonic stromal cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Rev* 2009;**5**:378–386.

47 Song L, Webb NE, Song Y, Tuan RS. Identification and functional analysis of candidate genes regulating mesenchymal stem cell self-renewal and multipotency. *Stem Cells* 2006;**24**:1707–1718.

48 Kaltz N, Ringe J, Holzwarth C, *et al.* Novel markers of mesenchymal stem cells defined by genome-wide gene expression analysis of stromal cells from different sources. *Exp Cell Res* 2010;**316**: 2609–2617.

49 Menicanin D, Bartold PM, Zannettino AC, Gronthos S. Genomic profiling of mesenchymal stem cells. *Stem Cell Rev Rep* 2009;**5**:36–50.

50 Loebel C, Czekanska EM, Bruderer M, *et al. In vitro* osteogenic potential of human mesenchymal stem cells is predicted by Runx2/Sox9 ratio. *Tissue Eng Part A* 2015;**21**(1–2):115–123. doi: 10.1089/ten.tea.2014.0096.

51 Phinney DG, Prockop DJ. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* 2007;**25** (11):2896–2902.

52 Both SK, van der Muijsenberg AJ, van Blitterswijk CA, *et al.* A rapid and efficient method for expansion of human mesenchymal stem cells. *Tissue Eng* 2007;**13**(1):3–9.

53 Stewart K, Monk P, Walsh S, *et al.* STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) as markers of primitive human marrow stromal cells and their more differentiated progeny: a comparative investigation *in vitro*. *Cell Tissue Res* 2003;**313**(3):281–290.

54 Jones EA, English A, Kinsey SE, *et al.* Optimization of a flow cytometry-based protocol for detection and phenotypic characterization of multipotent mesenchymal stromal cells from human bone marrow. *Clin Cytom* 2006;**70**(6):391–399.

55 Gronthos S, Zannettino AC, Hay SJ, *et al.* Molecular and cellular characterisation of highly purified stromal cells derived from human bone marrow. *J Cell Sci* 2003;**116**(Pt 9):1827–1835.

56 Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* 1991;**78**(1):55–62.

57 Whitney MJ, Lee A, Ylostalo J, *et al.* Leukemia inhibitory factor secretion is a predictor and indicator of early progenitor status in adult bone marrow stromal cells. *Tissue Eng Part A* 2009;**15** (1):33–44.

58 Turner S, Balain B, Caterson B, *et al.* Viability, growth kinetics and stem cell markers of single and clustered cells in human intervertebral discs: implications for regenerative therapies. *Eur Spine J* 2014;**23**(11):2462–2472.

59 Halfon S, Abramov N, Grinblat B, Ginis I. Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cell Dev* 2011;**20**(1):53–66.

60 Rasini V, Dominici M, Kluba T, *et al.* Mesenchymal stromal/stem cells markers in the human bone marrow. *Cytotherapy* 2013; **15**(3):292–306.

61 Fekete N, Rojewski MT, Fürst D, *et al.* GMP-compliant isolation and large-scale expansion of bone marrow-derived MSC. *PLoS ONE* 2012;**7**:e43255.

62 Cox G, Boxall SA, Giannoudis PV, *et al.* High abundance of $CD271^+$ multipotential stromal cells (MSCs) in intramedullary cavities of long bones. *Bone* 2012;**50**:510–517.

63 Tormin A, Li O, Brune JC, *et al.* CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with *in situ* localization. *Blood* 2011;**117**(19):5067–5077.

64 Blocki A, Wang Y, Koch M, *et al.* Not all MSCs can act as pericytes: functional in vitro assays to distinguish pericytes from other mesenchymal stromal cells in angiogenesis. *Stem Cell Dev* 2013;**22**(17):2347–2355.

65 Martinez C, Hofmann TJ, Marino R, *et al.* Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. *Blood* 2007;**109** (10):4245–4248.

66 Morikawa S, Mabuchi Y, Kubota Y, *et al.* Prospective identification, isolation and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J Exp Med* 2009; **206**,2483–2496.

67 Nakamura Y, Arai F, Iwasaki H, *et al.* Isolation and characterization of endosteal niche cell populations that regulate hematopoietic stem cells. *Blood* 2010;**116**:1422–1432.

68 Méndez-Ferrer S, Michurina TV, Ferraro F, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

69 Gronthos S, Franklin DM, Leddy HA, *et al.* Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 2001;**189**:54–63.

70 Yoshimura K, Shigeura T, Matsumoto D, *et al.* Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J Cell Physiol* 2006;**208**:64–76.

71 Mitchell J, McIntosh K, Zvonic S, *et al.* Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006;**24**:376–385.

72 Kinnaird T, Stabile E, Burnett MS, *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004;**94**(5):678–685.

73 Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells in vitro: effects of dexamethasone and IL-1 alpha. *J Cell Physiol* 1996; **166**(3):585–592.

74 Hsiao ST, Dilley RJ, Dusting GJ, Lim SY. Ischemic preconditioning for cell-based therapy and tissue engineering. *Pharmacol Ther* 2014;**142**(2):141–153.

75 Da Silva Meirelles S, Fontes AM, Covas DT, Caplan AI. Mechanisms involved in the therapeutic properties of mesenchymal stromal cells. *Cytokine Growth Factor Rev* 2009;**20**(5–6):419–427.

76 Mizuno H, Tobita M, Uysal AC. Concise review: adipose-derived stem cells as a novel tool for future regenerative medicine. *Stem Cells* 2012;**30**:804–810.

77 Yen BL, Yen ML, Hsu PJ, *et al.* Multipotent human mesenchymal stromal cells mediate expansion of myeloid-derived suppressor cells via hepatocyte growth factor/c-Met and STAT3. *Stem Cell Reports* 2013;**1**(2):139–151.

78 Ryan JM, Barry F, Murphy JM, Mahon BP. Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stromal cells. *Clin Exp Immunol* 2007; **149**(2):353–363.

79 Ortiz LA, Dutreil M, Fattman C, *et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stromal cells during lung injury. *Proc Natl Acad Sci U S A* 2007;**104**(26):11002–11007.

80 Milner CM, Higman VA, Day AJ. TSG-6: a pluripotent inflammatory mediator? *Biochem Soc Trans* 2006;**34**(Pt 3):446–450.

81 Crigler L, Robey RC, Asawachaicharn A, *et al.* Human mesenchymal stromal cell subpopulations express a variety of neuroregulatory molecules and promote neuronal cell survival and neuritogenesis. *Exp Neurol* 2006;**198**(1):54–64.

82 Li Y, Chen J, Chen XG, *et al.* Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* 2002;**59**(4):514–523.

83 Gui C, Wang JA, He AN, *et al.* Expression of heregulin and ErbB receptors in mesenchymal stem cells. *Chin Med J (Engl)* 2008; **121**(2):155–160.

84 Wilkins A, Compston A. Trophic factors attenuate nitric oxide mediated neuronal and axonal injury *in vitro*: roles and interactions of mitogen-activated protein kinase signalling pathways. *J Neurochem* 2005;**92**(6):1487–1496.

85 Bara JJ, McCarthy HE, Humphrey E, *et al.* Bone marrow-derived mesenchymal stem cells become antiangiogenic when chondrogenically or osteogenically differentiated: implications for bone and cartilage tissue engineering. *Tissue Eng Part A* 2014;**20**(1–2): 147–159.

86 Potapova IA, Gaudette GR, Brink PR, *et al.* Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells *in vitro*. *Stem Cells* 2007;**25**(7): 1761–1768.

87 Hoch AI, Binder BY, Genetos DC, *et al.* Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PloS One* 2012;**7**(4):e35579.

88 Liu H, Liu S, Li Y, *et al.* The role of SDF-1–CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. *PloS One* 2012;**7** (4):e34608.

89 Hu X, Yu SP, Fraser JL, *et al.* Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg* 2008;**135**(4):799–808.

90 Bartmann C, Rohde E, Schallmoser K, *et al.* Two steps to functional mesenchymal stromal cells for clinical application. *Transfusion* 2007;**47**(8):1426–1435.

91 Neuhuber B, Swanger SA, Howard L, *et al.* Effects of plating density and culture time on bone marrow stromal cell characteristics. *Exp Hematol* 2008;**36**(9):1176–1185.

92 Baer PC, Griesche N, Luttmann W, *et al.* Human adipose-derived mesenchymal stem cells in vitro: evaluation of an optimal expansion medium preserving stromalness. *Cytotherapy* 2010;**12**(1): 96–106.

93 Martin I, Muraglia A, Campanile G, *et al.* Fibroblast growth factor-2 supports ex vivo expansion and maintenance of osteogenic

precursors from human bone marrow. *Endocrinology* 1997; **138**(10):4456–4462.

94 Bianchi G, Banfi A, Mastrogiacomo M, *et al.* Ex vivo enrichment of mesenchymal cell progenitors by fibroblast growth factor 2. *Exp Cell Res* 2003;**287**(1):98–105.

95 Solchaga LA, Penick K, Porter JD, *et al.* FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* 2005;**203**(2):398–409.

96 Bocelli-Tyndall C, Zajac P, Di Maggio N, *et al.* Fibroblast growth factor 2 and platelet-derived growth factor, but not platelet lysate, induce proliferation-dependent, functional class II major histocompatibility complex antigen in human mesenchymal stem cells. *Arthritis Rheum* 2010;**62**(12):3815–3825.

97 Gharibi B, Hughes FJ. Effects of medium supplements on proliferation, differentiation potential, and *in vitro* expansion of mesenchymal stem cells. *Stem Cells Transl Med* 2012;**1**(11):771–782.

98 Spees JL, Gregory CA, Singh H, *et al.* Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stromal cells for cell and gene therapy. *Mol Ther* 2004;**9**:747–756.

99 Hemeda H, Giebel B, Wagner W. Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells. *Cytotherapy* 2014;**16**:170–180.

100 Tonti GA, Mannello F. From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera? *Int J Dev Biol* 2008;**52**(8):1023–1032.

101 Shahdadfar A, Fronsdal K, Haug T, *et al. In vitro* expansion of human mesenchymal stromal cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells* 2005;**23**(9):1357–1366.

102 Fekete N, Gadelorge M, Fürst D, *et al.* Platelet lysate from whole blood-derived pooled platelet concentrates and apheresis-derived platelet concentrates for the isolation and expansion of human bone marrow mesenchymal stromal cells: production process, content and identification of active components. *Cytotherapy* 2012;**14**:540–554.

103 Kinzebach S, Dietz L, Klüter H, *et al.* Functional and differential proteomic analyses to identify platelet derived factors affecting *ex vivo* expansion of mesenchymal stromal cells. *BMC Cell Biol* 2013;**14**:48.

104 Vogel JP, Szalay K, Geiger F, *et al.* Platelet-rich plasma improves expansion of human mesenchymal stem cells and retains differentiation capacity and *in vivo* bone formation in calcium phosphate ceramics. *Platelets* 2006;**17**:462–469.

105 Lange C, Cakiroglu F, Spiess AN, *et al.* Accelerated and safe expansion of human mesenchymal stromal cells in animal serum-free medium for transplantation and regenerative medicine. *J Cell Physiol* 2007;**213**:18–26.

106 Mishra A, Tummala P, King A, *et al.* Buffered platelet-rich plasma enhances mesenchymal stem cell proliferation and chondrogenic differentiation. *Tissue Eng Part C Methods* 2009;**15**(3):431–435.

107 Goedecke A, Wobus M, Krech M, *et al.* Differential effect of platelet-rich plasma and fetal calf serum on bone marrow-derived human mesenchymal stromal cells expanded *in vitro*. *J Tissue Eng Regen Med* 2011;**5**:648–654.

108 Bieback K, Hecker A, Kocaomer A, *et al.* Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells* 2009;**27**(9):2331–2341.

109 Schallmoser K, Bartmann C, Rohde E, *et al.* Replicative senescence-associated gene expression changes in mesenchymal stromal cells are similar under different culture conditions. *Haematologica* 2010;**95**:867–874.

110 Ben Azouna N, Jenhani F, Regaya Z, *et al.* Phenotypic and functional characteristics of mesenchymal stromal cells from bone marrow: comparison of culture using different media supplemented with human platelet lysate or fetal bovine serum. *Stem Cell Res Ther* 2012;**3**:6. doi: 10.1186/scrt97.

111 Kakudo N, Minakata T, Mitsui T, *et al.* Proliferation-promoting effect of platelet-rich plasma on human adipose-derived stem cells and human dermal fibroblasts. *Plast Reconstr Surg* 2008;**122**: 1352–1360.

112 Cholewa D, Stiehl T, Schellenberg A, *et al.* Expansion of adipose mesenchymal stromal cells is affected by human platelet lysate and plating density. *Cell Transplant* 2011;**20**:1409–1422.

113 Fekete N, Rojewski M, Lotfi R, Schrezenmeier H. Essential components for *ex vivo* proliferation of mesenchymal stromal cells. *Tissue Eng Part C Methods* 2014;**20**:129–139.

114 Lohmann M, Walenda G, Hemeda H, *et al.* Donor age of human platelet lysate affects proliferation and differentiation of mesenchymal stem cells. *PloS One* 2012;**7**:e37839.

115 Chase LG, Lakshmipathy U, Solchaga LA, *et al.* A novel serum-free medium for the expansion of human mesenchymal stem cells. *Stem Cell Res Ther* 2010;**1**(1):8. doi: 10.1186/scrt8.

116 Miwa H, Hashimoto Y, Tensho K, *et al.* Xeno-free proliferation of human bone marrow mesenchymal stromal cells. *Cytotechnology* 2012;**64**(3):301–308.

117 Gottipamula S, Ashwin KM, Muthigi MS, *et al.* Isolation, expansion and characterization of bone marrow-derived mesenchymal stromal cells in serum-free conditions. *Cell Tissue Res* 2014; **356**(1):123–135.

118 Al-Saqi SH, Saliem M, Asikainen S, *et al.* Defined serum-free media for *in vitro* expansion of adipose-derived mesenchymal stem cells. *Cytotherapy* 2014;**16**(7):915–926.

119 Basciano L, Nemos C, Foliguet B, *et al.* Long term culture of mesenchymal stromal cells in hypoxia promotes a genetic program maintaining their undifferentiated and multipotent status. *BMC Cell Biol* 2011;**12**:12. doi: 10.1186/1471-2121-12-12.

120 Dos Santos F, Andrade PZ, Boura JS, *et al. Ex vivo* expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. *J Cell Physiol* 2010; **223**:27–35.

121 Boyette LB, Creasey OA, Guzik L, *et al.* Human bone marrow-derived mesenchymal stem cells display enhanced clonogenicity but impaired differentiation with hypoxic preconditioning. *Stem Cells Transl Med* 2014;**3**:241–254.

122 Choi JR, Pingguan-Murphy B, Wan Abas WA, *et al.* Impact of low oxygen tension on stemness, proliferation and differentiation potential of human adipose-derived stem cells. *Biochem Biophys Res Commun* 2014;**448**(2):218–224.

123 Valorani MG, Montelatici E, Germani A, *et al.* Pre-culturing human adipose tissue mesenchymal stem cells under hypoxia increases their adipogenic and osteogenic differentiation potentials. *Cell Prolif* 2012;**45**:225–238.

124 Pattappa G, Thorpe SD, Jegard NC, *et al.* Continuous and uninterrupted oxygen tension influences the colony formation

and oxidative metabolism of human mesenchymal stem cells. *Tissue Eng Part C Methods* 2013;**19**:68–79.

125 Merceron C, Vinatier C, Portron S, *et al.* Differential effects of hypoxia on osteochondrogenic potential of human adipose-derived stromal cells. *Am J Physiol Cell Physiol* 2010;**298**:C355–C364.

126 Wagegg M, Gaber T, Lohanatha FL, *et al.* Hypoxia promotes osteogenesis but suppresses adipogenesis of human mesenchymal stromal cells in a hypoxia-inducible factor-1 dependent manner. *PloS One* 2012;**7**:e46483.

127 Binder BY, Sagun JE, Leach JK. Reduced serum and hypoxic culture conditions enhance the osteogenic potential of human mesenchymal stem cells. *Stem Cell Rev* 2015;**11**(3):387–393.

128 Rosová I, Dao M, Capoccia B, *et al.* Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 2008;**26**:2173–2182.

129 Seeger FH, Tonn T, Krzossok N, *et al.* Cell isolation procedures matter: a comparison of different isolation protocols of bone marrow mononuclear cells used for cell therapy in patients with acute myocardial infarction. *Eur Heart J* 2007;**28**(6):766–772.

130 Schachinger V, Erbs S, Elsasser A, *et al.* Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J* 2006;**27**(23):2775–2783.

131 Lunde K, Solheim S, Aakhus S, *et al.* Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006;**355**(12):1199–1209.

132 Assmus B, Tonn T, Seeger FH, *et al.* Red blood cell contamination of the final cell product impairs the efficacy of autologous bone marrow mononuclear cell therapy. *J Am Coll Cardiol* 2010; **55**(13):1385–1394.

133 Mouquet F, Lemesle G, Delhaye C, *et al.* The presence of apoptotic bone marrow cells impairs the efficacy of cardiac cell therapy. *Cell Transplant* 2011;**20**(7):1087–1097.

134 Sart S, Tsai AC, Li Y, Ma T. Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications. *Tissue Eng Part B Rev* 2013;**20** (5):365–380.

135 Frauenschuh S, Reichmann E, Ibold Y, *et al.* A microcarrier-based cultivation system for expansion of primary mesenchymal stem cells. *Biotechnol Prog* 2007;**23**(1):187–193.

136 Carmelo JG, Fernandes-Platzgummer A, Cabral JM, da Silva CL. Scalable ex vivo expansion of human mesenchymal stem/stromal cells in microcarrier-based stirred culture systems. *Methods Mol Biol* 2015;**1283**:147–159.

137 Hupfeld J, Gorr IH, Schwald C, *et al.* Modulation of mesenchymal stromal cell characteristics by microcarrier culture in bioreactors. *Biotechnol Bioeng* 2014;**111**(11):2290–2302.

138 Kuan-Liang Chen A, Chew YK, Tan HY, *et al.* Increasing efficiency of human mesenchymal stromal cell culture by optimization of microcarrier concentration and design of medium feed. *Cytotherapy* 2015;**17**(2):163–173.

139 Santos F, Andrade PZ, Abecasis MM, *et al.* Toward a clinical-grade expansion of mesenchymal stem cells from human sources: a microcarrier-based culture systromal under xeno-free conditions. *Tissue Eng Part C Methods* 2011;**17**(12):1201–1210.

140 Braccini A, Wendt D, Jaquiery C, *et al.* Three-dimensional perfusion culture of human bone marrow cells and generation of osteoinductive grafts. *Stem Cells* 2005;**23**(8):1066–1072.

141 Scaglione S, Braccini A, Wendt D, *et al.* Engineering of osteoinductive grafts by isolation and expansion of ovine bone marrow stromal cells directly on 3D ceramic scaffolds. *Biotechnol Bioeng* 2006;**93**(1):181–187.

142 Papadimitropoulos A, Piccinini E, Brachat S, *et al.* Expansion of human mesenchymal stromal cells from fresh bone marrow in a 3D scaffold-based system under direct perfusion. *PloS One* 2014; **9**(7):e102359.

143 Claros S, Rodriguez-Losada N, Cruz E, *et al.* Characterization of adult stem/progenitor cell populations from bone marrow in a three-dimensional collagen gel culture system. *Cell Transplant* 2012;**21**(9):2021–2032.

CHAPTER 8

# The effect of three-dimensional aggregates on the biology of mesenchymal stromal cells

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## 8.1 Three-dimensional multicellular aggregates

Spherical aggregates of mammalian cells have been widely used in biological research for many decades. A variety of names, such as aggregates, spheroids, spheres (e.g., neurospheres, cardiospheres), have been used to describe the phenomenon in which single cells in suspension spontaneously coalesce and form stable and spherical aggregates in culture composed of multiple cells. In the pioneering studies by Holtfreter on tissue morphogenesis, spherical aggregates of embryonic cells were obtained by culturing embryonic explants or single cells on nonadherent agar as a substratum or on a shaking device to reduce cell–substratum interactions [1,2]. These techniques were later used by Moscona to obtain multicellular tumor spheroids as an *in vitro* model to understand the formation and invasive potential of tumor cells [3]. The approaches employed in these studies, such as using nonadherent culture surfaces and/or a gyratory shaker to maintain individual cells in suspension in order to encourage aggregation and prevent cell attachment to surfaces, have been the major methods in the subsequent studies on multicellular aggregates in tissue morphogenesis, tumor malignancy and response to therapy, and in somatic cell reprogramming [4,5]. The persistent interest in three-dimensional (3D) cellular aggregates stems from the unique biological properties exhibited by 3D cellular aggregates compared with their two-dimensional (2D) adherent counterparts. It has been observed in neural stem cells that the *in vitro* formation of multicellular spheroids by nestin-expressing neural cells on a nonadhesive substratum (i.e., neurospheres) confers upon the cells many defining properties of

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stem cells. This has led to the use of cell aggregations to define and measure neural stem cells. In these studies the presence, frequency, and dimension of neurospheres have been used as a colony-forming assay to assess the ability of the cells to yield stem cells as well as to assess their clonality and mitogenic potential. Further studies, however, suggest that the ability to form floating cell clusters on nonadherent surfaces or in suspension is not limited to neural cells but shared by virtually all dividing cells in many tissues and organs with intercellular adhesive properties, including the ear, skin, pancreas, and mammary gland [6–10]. In addition to being a defining feature of embryonic stem cells, the persistent proliferation of cells in suspension indicates the loss of anchorage dependence, which is also a hallmark of tumor cells. On the other hand, differentiated somatic cells also have the ability to coalesce and form 3D aggregates and a 3D environment that drastically changes their phenotypic properties. Because of anchorage dependence, somatic cells typically do not exhibit extensive proliferative properties in 3D aggregates and instead they form 3D aggregates based on cell–cell interactions mediated by the extracellular matrix (ECM). For example, multicellular spheroids of endothelial cells consist of a surface layer of differentiated cells and a core of unorganized cells undergoing apoptosis [11]. Depending on cell type, formation of multicellular aggregates also significantly alters cell phenotypic properties. Thus, formation of multicellular aggregates promotes fibroblast reprogramming and acquisition of stemness properties [5]. Although *in vitro* self-assembly of multicellular aggregates is a common phenomenon for many cell types, the mechanism underling the self-assembly varies, and its subsequent impact on cell property is cell-type specific and regulated by extrinsic environmental factors as well as intrinsic cell susceptibility to these environmental factors.

## 8.2 Three-dimensional aggregates of mesenchymal stromal cells

Mesenchymal *stromal* cells (MSCs), defined as having the ability for colony-forming unit–fibroblast formation, plastic adherence, trilineage mesodermal differentiation potential, and expression of characteristic surface markers, were originally derived from bone marrow but have now been isolated from multiple tissues and organs, including adipose tissue, Wharton's jelly, amniotic fluid and dental pulp. The *in vivo* mesenchymal *stem* cell identity and developmental origin is believed to be a skeletal stem cell that not only can self-renew into daughter mesenchymal stem cells but also can give rise to mesenchymal *stromal* progenitor cells for tissues of the musculo-skeletal system, and, in addition and importantly, can support hematopoiesis [12] (and see Chapters 32 and 47). Studies have demonstrated that transplantation of the nonmineralized cartilage pellets formed by human bone marrow MSCs (BM-MSCs) *ex vivo* can generate complete ossicles with normal bone marrow structures in the absence of exogenous scaffolds, demonstrating their multipotency [13]. *In vitro* MSCs are clonogenic and are able to initiate clonal growth in a density-dependent fashion to form colony-forming unit–fibroblasts (CFU-Fs), a defining property of MSCs. Another feature of MSCs is their strong plastic adhesion property, which allowed their separation from hematopoietic cells in their original discovery by Friedenstein [14]. However, MSCs in adherent plastic culture are sensitive to culture density and change their population characteristics during expansion, resulting in a remarkable population heterogeneity within a small number of population doublings. These results suggest that MSCs in *in vitro* cultures are not equivalent to the culture of "stromal cells" and that factors in their environmental milieu, such as oxygen tension, substrate stiffness, and cell–cell interactions, significantly influence MSC properties. To optimally translate MSCs into a therapeutic agent there is a need to understand the impact of environmental factors on MSC fate and properties.

*In vitro* formation of 3D aggregates has emerged as an important approach by which to study MSC biology. It may also be important for augmenting their biological properties for therapeutic applications. During development, bone, cartilage, and bone marrow stroma differentiate from, and are downstream of, mesenchymal condensation, which is a critical transitional stage in the development of mesenchymal tissues and which precedes cartilage during embryonic development. It is regulated by cell adhesion and the ECM [15]. Condensation occurs when a previously dispersed population of cells gathers together to differentiate into a single cell/tissue type such as cartilage, bone, muscle, tendon, kidney, or lung and is the earliest stage during organ formation when tissue-specific genes are upregulated [15].

The *in vitro* ability of MSCs to form 3D multicellular aggregates free of exogenous scaffolds was first studied in pellet culture of bone-marrow-derived progenitor cells during their differentiation into chondrocytes [16]. In the presence of transforming growth factor-β3 the formation of tightly packed cellular aggregates eventually developed into well-stratified cartilage and the condensed mesenchymal bodies were able to fuse and form mechanically functional cartilaginous tissue interfacing with bone [17]. These studies suggested that *in vitro* MSC aggregation mimics mesenchymal condensation and is integral to establishing the physiologic milieu for early skeletal development.

Subsequent studies have shown broad changes in MSC properties upon 3D aggregation compared with their 2D counterparts. It was reported that scaffold-free culture of MSCs as spheroids preserved multilineage potential and enhanced their differentiation into multiple lineages while downregulating the genes maintaining the self-renewal phenotypes of MSCs [18,19]. The 3D aggregation also significantly enhanced their trophic effects and therapeutic outcomes in various disease models. Thus, it is important to understand the mechanism of MSC self-assembly and its role in functional enhancement and in animal models, as well as to develop technology for the production of 3D aggregates.

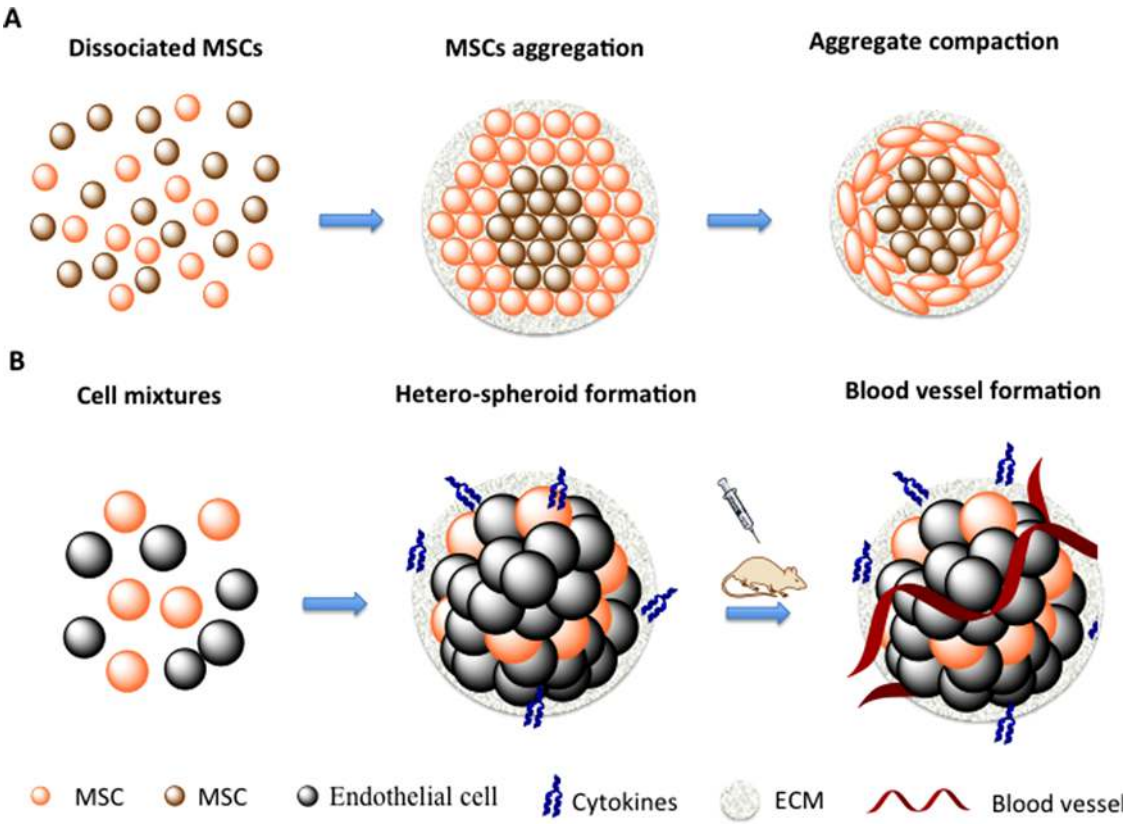



**Figure 8.1** MSCs 3D aggregate self-assembly and MSC heterospheroid formation. (A) MSC aggregation process. Dissociated MSCs first form loosely packed aggregates in which cells with more cadherin cluster in the interior of the aggregate, whereas cells with less cadherin are aligned along the exterior of the aggregate. Aggregate formation is followed by reorganization of the ECM and actomyosin-mediated compaction that leads to mechanical polarization and cell spreading at the exterior of the aggregate. (B) Formation of a heterospheroid comprised of MSCs and endothelial cells and their application in cardiac vascularization. Dissociated MSC and endothelial cell mixtures form 3D heterospheroid aggregates *in vivo* via cell–cell adhesion molecules. The heterospheroids exhibit more potent angiogenic properties *in vivo* because of increased secretion of ECM, abundant antiapoptotic and proangiogenic cytokines, and increased cell survival.

## 8.3 Mechanism of mesenchymal stromal cells self-assembly into three-dimensional aggregates

### 8.3.1 Cell–cell contact

The differential adhesion hypothesis suggested that embryonic cells have liquid-like behavior and are able to sort out from each other during the developmental stage to achieve their normal histological pattern. This cell segregation phenomenon is regulated by tissue surface tension arising from differences in intercellular adhesiveness. A cell aggregate of lower surface tension tends to envelop one of higher surface tension to which it adheres, and the surface tension is linearly correlated with cell cadherin expression level [20]. MSCs share similarity to embryonic cells in terms of liquid-like behavior and readily self-assemble into 3D aggregates in suspension culture, as depicted in Figure 8.1A.

While the role of cadherin in regulating MSC cytoskeleton tension during aggregate formation is not conclusive, E-cadherin is reported to be a key inducer mediating human umbilical cord blood-derived MSCs (UCB-MSCs) to form 3D aggregates, among several calcium-dependent adhesion molecules [21]. In addition, cadherin-mediated adhesion is intrinsically mechanosensitive and is a crucial component of the cells' tension-sensing apparatus [22]. The observations that human adipose-tissue-derived stromal cells (ADSCs) can self-assemble into 3D aggregates in serum-free media and that the switch between N-cadherin and OB-cadherin in rat ADSCs in 3D aggregates is substrate-dependent further indicate that cadherin is not only involved in MSC aggregate self-assembly but is also highly dynamic and tightly regulated during aggregate rearrangement [23,24].

### 8.3.2 Extracellular matrix and the cytoskeleton

Apart from cadherin, ECM proteins also play important roles in aggregate formation and compaction. During hepatoma spheroid formation, ECM fibers act as a long chain linker for the attachment of dispersed single cells to form loose aggregations through the binding of integrin [25]. In addition, integrin-mediated cell–ECM connections are crucial for tissue

homeostasis during embryonic development as well as for wound healing [26]. The study of an α5 integrin-transfected Chinese hamster ovary cell line showed that the interplay between α5 integrin and soluble fibronectin contributes significantly to tissue cohesion and mediates a shift from liquid to elastic behavior of 3D cellular aggregates, demonstrating the importance of integrin and ECM interaction in mediating cell aggregate mechanical properties, as well as in segregation between cells [26]. Indeed, contiguous scanning electron microscopy and transmission electron microscopy analysis of 3D aggregates of human MSCs over a 7-day period revealed that cell aggregates undergo dynamic compaction with changes in cellular architecture and mechanical properties [27]. Both cell-secreted ECM and incorporated exogenous ECM have been reported to enhance the mechanical properties (e.g., stiffness) of human MSCs in 3D aggregates [18,24]. Integrin expression is also found to be altered in cells derived from human MSC spheroids, in that cells from human MSC spheroids upregulate expression of the integrin α2 subunit but suppress that of the integrin α4 subunit. However, the expression of these integrin subunits can be restored when the cells are cultured in monolayer culture [28], indicating the important role of integrin in cytoskeleton reorganization during aggregate compaction [29].

Along with changes in cadherin and integrin expression, 3D self-assembly and subsequent compaction reduce MSC size and alter cell morphology and cytoskeleton tension in a spatially dependent manner [30,31]. The significant size and morphological changes of MSCs in 3D aggregates require effective rearrangement and adaptation of the actomyosin network. Actin filaments, known to form stress fibers in response to external biomechanical stress in 2D adherent culture, control cell morphology in 2D adherent culture and regulate the contraction and fusion of 3D embryoid bodies [32,33]. In MSC 3D aggregates, adaptive changes in actin spatial distribution lead to mechanical polarization with cells at the interior of the aggregates having lower cytoskeleton stress compared with cells at the exterior of the aggregates [31,34]. Thus, actin plays a major role in mediating the compaction of 3D MSC aggregates and their interactions with the external environment. Unpublished data from our laboratory have shown that interfering with actin-mediated contractility by cytochalasin D, nocodazole, lysophosphatidic acid, and ROCK inhibitor Y-27632 not only influenced human MSC 3D aggregation and compaction, but also fusion between the adjacent aggregates and their spreading on an adherent surface. Cytochalasin D is a cell-permeable potent inhibitor of actin polymerization. Nocodazole is an antineoplastic agent that exerts its effect on cells by interfering with the polymerization of microtubules. Microtubules are one type of fiber that constitutes the cytoskeleton. Lysophosphatidic acid is a phospholipid derivative that can act as a signaling molecule and is a potent mitogen owing to its activation of three high-affinity G-protein-coupled receptors. ROCK inhibitor Y-27632 is a cell-permeable, highly potent and selective inhibitor of Rho-associated, coiled-coil-containing protein kinase (ROCK).

### 8.3.3 Mesenchymal stromal cells heterospheroids

By taking advantage of their trophic and adhesive properties, MSCs have been used to form heterospheres with other cell types to augment their therapeutic effects (Figure 8.1B). For example, heterospheroids composed of rat islet cells and human MSCs have been successfully engrafted in the same area of liver as islet cells after transplantation via the portal vein, resulting in increased angiogenesis and decreased apoptosis of islets compared with transplantation of islets alone [35]. Similarly, the transplantation of 3D composite aggregates of co-cultured human umbilical vein endothelial cells (HUVECs) and UCB-MSCs considerably enhanced the vascular densities and the blood flow recovery in the ischemic myocardium in rats with myocardial infarction compared with their dissociated counterparts [36]. As with 3D MSC aggregates, both cadherin-mediated cellular adhesion and integrin-mediated cytoskeleton tension play important roles in regulating 3D aggregation and compaction of heterospheres. In MSC/islet cell heterospheres treated with the ROCK inhibitor Y-27632, cell segregation was observed within 3 days of aggregation in which aggregates sorted into core–shell structures with islet-derived cells occupying the exterior while MSCs occupied the core [37]. Similarly, by changing integrin signaling and cell–cell interaction by altering the biomaterial substrate, the cellular pattern of self-assembled MSC and endothelial progenitor cell (EPC) aggregates exhibited different morphologies, including randomly mixed, bumped, or concentric spheroids, in which EPCs were wrapped by MSCs with a well-aligned organization [23]. Further understanding the mechanisms underlying segregation of MSCs within heterospheroids into certain patterns will be important for the fabrication of MSCs and other cell mixtures for the production of complex tissue patterns.

## 8.4 Mechanisms of aggregate-mediated mesenchymal stromal cell functional enhancement

MSCs were initially studied as stromal cells in tissue engineering and regeneration research because of their potential for differentiating into a number of tissues of mesodermal lineage, including bone and cartilage. Additionally, MSCs have strong tropic (migratory) effects and are a significant source of ECM components. Through these mechanisms, administered MSCs can establish a niche that stimulates the propagation and differentiation of endogenous stromal cells. MSCs are also highly responsive to the macromolecular milieu and can engage in extensive crosstalk triggered by signals from injured tissues. The secretion of many proteins by MSCs plays an important role in this because some of the factors can downmodulate excessive and harmful inflammation; MSCs can also play an immunomodulatory role on both the innate immune response (see Chapter 33) and the adaptive immune response (see Chapter 34) and can

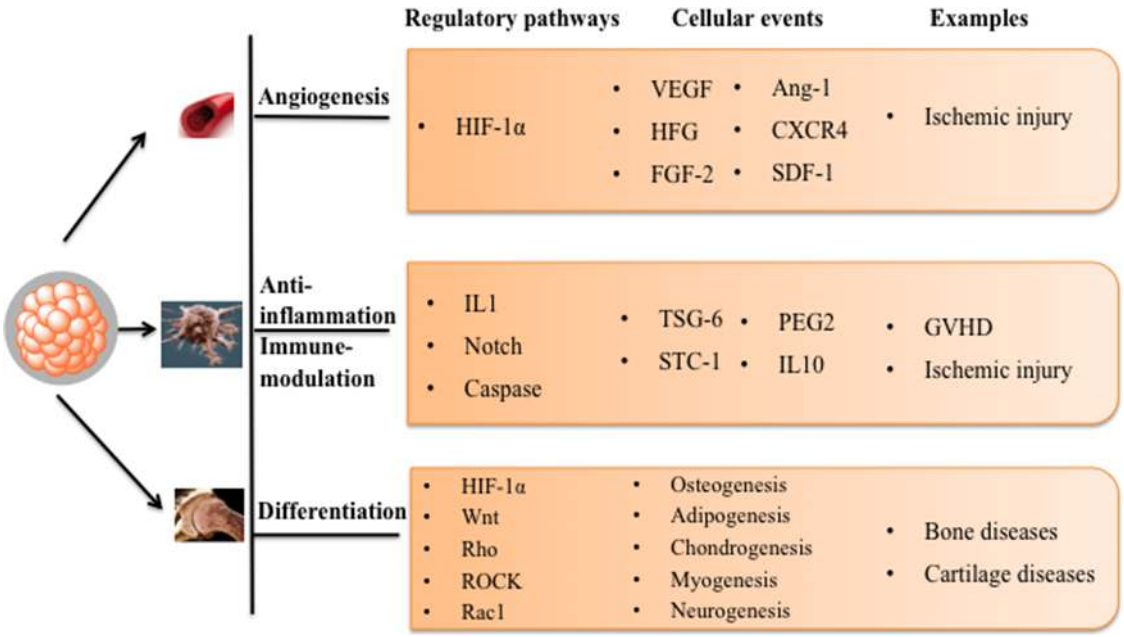



**Figure 8.2** Summary of the therapeutic properties and regulatory pathways of MSC 3D aggregates.

establish a macromolecular milieu that promotes tissue regeneration. Thus, preserving the mesodermal lineage differentiation potential, the secretory function and the migratory functions of MSCs are important. Studies have shown that MSC self-assembly into 3D aggregates significantly influences these properties by regulating the aggregation-associated changes in inter- and intra-cell interactions, cytoskeleton and cell morphology changes, and alterations in the molecular milieu of the 3D aggregates (Figure 8.2).

### 8.4.1 Role of cell adhesion molecules in the fate decision of mesenchymal stromal cell three-dimensional aggregates

Cellular adhesion molecules (CAMs) such as the cadherins and connexins are among key factors governing the transition from single cells to tissues during tissue development. The expression of adhesion molecules often undergoes change during the *in vitro* differentiation of pluripotent aggregates; for example, the high expression of E-cadherin in undifferentiated pluripotent embryonic stem cells decreases upon differentiation, concomitant with differential expression of many other cell adhesion molecules during lineage commitment [33]. MSC 3D aggregates are also sensitive to regulation by adhesion molecules. Cell–cell contact or culture surface profoundly impact MSC fate decision [38]. By controlling cell–cell contact on RGD-coated micropatterned material surfaces, osteogenic and adipogenic differentiation by MSCs were regulated by cell-cell contact. (RGD is an abbreviation for the tripeptide arginylglycylaspartic acid and is composed of L-arginine, glycine, and L-aspartic acid. The sequence is a common element in cellular recognition processes.) Paired or aggregated cells exhibited significantly enhanced differentiation capacity compared with isolated cells, and the extent of differentiation appeared to be linearly related to the extent of contact factor [39]. A dense mass of aggregated MSCs with strong cell–cell interaction promotes the immediate differentiation of MSCs into chondroblasts that can be further enhanced through the addition of cellular interaction enhancement components such as poly-L-lysine [40]. In addition to the extent of cell–cell contact, MSC differentiation *in vitro* is also correlated with a specific pattern of cadherin molecules, as selectively enhanced expression of N-cadherin, OB-cadherin, and R-cadherin were observed during differentiation of MSCs to osteogenesis, myogenesis, and adipogenesis respectively [41]. In 3D spheroids of rat adipose-tissue-derived MSCs there was differential upregulation of N-cadherin and OB-cadherin upon chondrogenic and osteogenic induction. This further suggests a differential association of CAMs by 3D MSC aggregates upon lineage commitment [42].

The regulatory effect of cell contact-mediated cadherin molecules on MSC 3D aggregate differentiation is regulated through transcriptional signaling. By manipulation of cellular adhesion patterns using different biomaterial substrates, MSC spheroids showed increased expression of Wnt and its downstream signaling, which correlated with MSC aggregate differentiation. It was shown that canonical Wnt3a-mediated signaling was more active for MSC spheroids differentiated osteogenically, while Wnt5a-mediated noncanonical Wnt signaling was more active for MSC spheroids that had undergone chondrogenic differentiation [43]. Adipose tissue-derived MSC spheroids cultured on chitosan membrane also showed increased expression of N-cadherin and Wnt-11 signaling and increased cardiomyogenic differentiation [42]. Thus, MSC 3D aggregates provide a microenvironment that has strong cell–cell contact and selectively

enriched cell adhesion molecules that can be utilized to control cell fate and recapture tissue development.

### 8.4.2 Effects of extracellular matrix, cytoskeleton, and morphology on mesenchymal stromal cell lineage commitment in three-dimensional aggregates

In addition to CAMs, 3D multicellular aggregates are also in contact with ECM. This is known to influence stromal cell fate and functions via a myriad of intercellular signaling pathways [22]. ECM and cells bind and interact through integrins that influence the intercellular actomyosin network via a ROCK-mediated pathway, which in turn controls matrix assembly, stabilization, and biomechanical properties of cells [34]. In 3D aggregates interactions between the ECM and the actomyosin network via integrins influence the biomechanical properties and cell fate decision of the aggregates. It has been reported that 3D aggregates of human ADSCs formed on chitosan films produced more extracellular molecules, such as laminin and fibronectin, compared with those in a monolayer culture. They readily differentiated into neuron and hepatocyte-like cells upon induction [44]. (Chitosan is a linear polysaccharide composed of randomly distributed β-(1–4)-linked D-glucosamine (the deacetylated unit) and *N*-acetyl-D-glucosamine (the acetylated unit). It is made by treating shrimp and other crustacean shells with the alkali sodium hydroxide).

Incorporating gelatin into 3D aggregates of human MSCs also changed aggregate stiffness and mechanical properties [18].

Approaches to modulate cell adhesion, morphology, and cell fate through manipulation of adhesive ligand properties or micropatterned substrates have also been extensively explored in monolayer cultures. Downstream signaling of the Rho-associated protein kinase (ROCK) pathway is often implicated in cytoskeletal reorganization and stiffness-mediated phenotypic changes [45]. MSCs undergo dramatic size changes within 3D aggregates. Cells derived from 3-day and 7-day aggregates showed significantly decreased cell size compared with monolayer MSCs [30]. In addition to changes of cell size, cell morphology also showed changes in spatial distribution. In 7-day aggregates, cells on the surface of the spheroids were more spread out and had less distinct boundaries between each other, whereas cells in the interior had a more irregular morphology [30]. The spatial differences in cell morphology within 3D aggregates of MSCs correlated well with differentiation ability: on induction of soluble factors permitting osteogenic and adipogenic differentiation, human MSCs at the edge of multicellular spheroids underwent osteogenic differentiation whereas those in the center of the aggregate underwent adipogenic differentiation, possibly via Ras homolog A (RhoA) signaling [31,46]. This differentiation pattern was further shown to result from different levels of cytoskeleton stress, where regions of high stress resulted in osteogenesis, whereas cells in regions of low stress differentiated to adipocytes [31]. Examination of cell morphology during BMP-2-induced cartilage formation of human MSC micromasses also demonstrated that the spatial patterns of cell distribution were closely associated with distinct cell fate and ECM-building ability [47]. As with MSCs in 2D adherent culture, MSC fate decision in 3D aggregates is thought to be regulated by RhoA and Rac1 (Ras-related C3 botulinum toxin substrate 1), which integrate signaling from cytoskeleton tension or crosstalk with adhesion molecules, thus highlighting the tight coupling between lineage commitment and changes in cell shape, cell-matrix adhesion, and cell–cell adhesion that occur during cell morphogenesis [46].

### 8.4.3 Role of molecular milieu and hypoxia-inducible factor activation

Compared with 2D adherent culture, 3D multicellular aggregates form diffusional barriers and create concentration gradients of oxygen, cell-secreted cytokines, and serum-borne macromolecules in the interior of the aggregates [48]. Of these molecules, restricted oxygen delivery and formation of a hypoxic core have been frequently cited as the primary factor for activation of hypoxia-inducible factor (HIF) and the subsequent upregulation of proangiogenic factors, proinflammatory cytokines, and increased resistance to oxidative stress [49–51]. Using ADSCs transfected with a fluorescence HIF probe, HIF activation in the ADSC aggregate was shown to be induced by controlling the size of the aggregates and that HIF-1 activity increased with increasing aggregate size and with decreasing external oxygen concentration [52]. However, modeling of the oxygen gradient in 3D human MSC aggregates indicated limited oxygen depletion and oxygen concentration far above the threshold for HIF activation in 2D adherent culture. Further study showed that HIF-1α expression is not strongly influenced by aggregate size but is correlated with the level of cell compaction after centrifugation [53]. Thus, reduced oxygen concentration may not be the sole factor for HIF activation in 3D aggregates. Although post-translational HIF regulation by hypoxia has been most frequently studied, there is growing evidence that HIF is also subject to transcriptional and posttranscriptional modification by non-hypoxic stimuli such as angiotensin II, thrombin, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), interleukin (IL)-6, IL-8, and glucose [54]. Since 3D aggregation of MSCs is a potent inducer of angiogenic factors and several ILs, the molecular milieu formed in the interior of the aggregates may also contribute to HIF upregulation. Thus, HIF activation in the 3D aggregates may be modulated by multiple environmental factors, including both hypoxic and nonhypoxic stimuli.

HIF activation in 3D MSC aggregates is a major mechanism for the enhanced secretion of angiogenic and antiapoptotic factors. For example, spheroid culture of human ADSCs upregulated HIF-1α expression with enhanced secretion of HGF, VEGF, and fibroblast growth factor 2 (FGF-2) [49]. Co-cultured

HUVEC/UCB-MSCs in 3D aggregates with different degrees of internal cell density also exhibited HIF-1α-mediated upregulation of VEGF, adrenomedullin, angiopoietin 1, stromal cell-derived factor 1 (SDF-1, also known as CXCL12), and chemokine motif C–X–C receptor 4 (CXCR4), suggesting enhanced angiogenesis, cell retention and homing ability [53]. (Adrenomedullin is a ubiquitously expressed peptide hormone that was initially isolated from a pheochromocytoma, a tumor of the adrenal medulla – hence its name). HGF, matrix metalloproteinase (MMP)-2 and MMP-14 are also seen to be significantly produced in human ADSCs, resulting in increased wound closure rate compared with monolayer cells in a diabetic animal model [55].

Apart from enhancing secretory functions, HIF activation also upregulates genes associated with stemness, including *OCT-4*, *SOX-2*, and *NANOG* in MSCs [50]. Of these, *OCT-4* expression is essential for maintaining an undifferentiated cell fate. Studies have shown that extended HIF-2α expression increased *OCT-4* mRNA and protein levels in embryoid bodies and is an upstream regulator of *OCT-4* expression in embryonic stem cells [56]. Similar findings on *OCT-4* upregulation via HIF-1α activation under hypoxic conditions, with a subsequent increase in neurogenic potential in response to environmental cues, have also been reported for ADSCs [57]. Thus, HIF activation resulting from a limitation of oxygen diffusion, or its interaction with the molecular milieu in the 3D aggregate, is an important factor in the multilineage potential observed in various types of MSC aggregates.

### 8.4.4 Metabolism changes in three-dimensional aggregates of mesenchymal stromal cells

Since multicellular aggregates create a heterogeneous microenvironment accompanied by size-dependent gradients of oxygen and nutrients, cells within a 3D aggregate may undergo metabolic changes that have an impact on their cellular properties. It has been demonstrated that malignant cell aggregates can adapt their metabolism to the specific environment conditions in a 3D aggregate by reducing their metabolic activity [58,59]. This contributed significantly to their viability and clonogenicity.

MSCs derived from normal gum (gingiva) tissue and cultured as multicellular spheroids were found to be arrested in a quiescent stage of the cell cycle with a significantly decreased number in S phase and more than 90% of cells in $G_0/G_1$ phases. An increased level of reactive oxygen species and antioxidants such as manganese superoxide dismutase were also observed in these aggregates [50]. Human dermal stromal cells cultured in 3D aggregates showed higher expression of dermal progenitor markers, including SOX2, Nanog, S100B, and enhanced expression of human telomerase reverse transcriptase [60]. S100 proteins are localized in the cytoplasm and nucleus of a wide range of cells and are involved in the regulation of a number of cellular processes, such as cell cycle progression and differentiation.

### 8.4.5 Enhanced anti-inflammatory properties of three-dimensional aggregates of mesenchymal stromal cells

Another interesting property of MSC 3D aggregate cultures is the self-activated release of anti-inflammatory cytokines, including tumor necrosis factor (TNF)-α-induced protein-6 (TSG-6), stanniocalcin-1 (STC-1), prostaglandin $E_2$ ($PGE_2$), and IL-10, which have been shown to suppress the activity of stimulated macrophages *in vitro* and *in vivo* [61–63]. Although human MSCs grown in a standard monolayer culture have been shown to improve cardiac function and decrease cardiac scarring in a murine model of acute myocardial infarction by secretion of the anti-inflammatory factor TSG-6, its secretion has to be activated by incubation with the inflammatory cytokine TNF-α for at least 24 h [64]. In the absence of inflammatory cytokines, 3D MSC aggregates can self-activate to secrete potent anti-inflammatory cytokines such as TSG-6 and $PGE_2$. The mechanisms by which they do this are under exploration. Culture medium appears to be one factor that turns on/off the expression and secretion of $PEG_2$ and TSG-6 [51,61,65]. Culture-medium-mediated control of the secretory function of MSC 3D aggregates is closely correlated with cell survival. It appears that culture medium that supports cell survival and reduces cell stress during 3D aggregation has a negative effect on secretory ability of the MSC aggregate. Bartosh *et al.* reported that the stress-activated signaling pathway of IL-1α/β is involved in the activation of the immune response of MSC aggregates, including the secretion of TSG-6 and STC-1, and that an independent contact-dependent Notch signaling pathway is required for secretion of $PGE_2$ production [63].

## 8.5 Bioreactor systems for three-dimensional aggregate production

### 8.5.1 Scale-up and dynamics of culture

Enhancement of the properties of human MSCs by 3D aggregate formation has motivated the development of bioreactor systems for aggregate production. This is due to their scalability and the ability to provide a physiochemical environment with tightly controlled culture parameters, such as oxygen tension, mechanical stresses, and nutrient gradients. Bioreactor systems for multicellular aggregate formation and production have already been developed and used for tumor spheroids, pluripotent stem cells, and neural stem cells [66–68]. Compared with these cell types, MSCs have a significantly lower proliferation potential in suspension, are highly adhesive, and have high sensitivity to mechanical stress. For example, MSCs form 3D aggregates primarily through cell-cell adhesion rather than by proliferation because they have limited proliferative ability in suspension.

MSC aggregates are highly adhesive to the external surface and have a strong tendency to coalesce into larger aggregates [51]. MSC aggregates also exhibit viscoelastic properties and are highly sensitive to external mechanical stresses. These properties strongly influence the initiation of MSC aggregation, aggregate

deformation, their biological properties, and consequently the design and operation of bioreactor systems to produce them in bulk.

In suspension, the initial process of multicellular aggregation depends primarily on cell–cell collision followed by cell–cell contact via intercellular adhesion and coalescence [69]. Owing to the low MSC survival in suspension cultures, bioreactors used in MSC aggregate production typically require preseeding of the aggregates formed in ultralow attachment culture dishes, hanging-drop plates, or by centrifugation in AggreWell™ plates [18,27]. Following the seeding of the preformed aggregates, the magnitude and profile of shear rate and shear stress influence the size of MSC aggregates [70–72]. This is likely due to higher force input interrupting cell–cell connections, thereby reducing coalescence and limiting aggregate size. At low shear, smaller MSC aggregates easily adhere to each other and coalesce into larger aggregates. When growing embryonic stem cells in culture, Mogi *et al.* reported an inverse correlation between the average diameters of embryoid bodies and the rotation rate in rotary orbital suspension culture [73]. This phenomenon can be explained by hydrodynamic analysis of the microcarrier bioreactor in which the Kolmogorov eddies decrease with the decrease of fluid viscosity or the increase of dissipation energy per unit mass [74,75]. Kolmogorov microscales are the smallest scales in turbulent flow. Very similar to Jossen's eddy analysis in microcarrier systems with turbulent flow, the higher energy input increases the turbulent eddy number but decreases the eddy diameter, thus generating directional forces to detach cells from aggregate surfaces [74]. However, if the shear stress is too high, the possibility of cell–cell collision increases concomitant with the decrease of collision time, which inhibits cell–cell contact and the cadherin accumulation in the cell–cell contact area. This prevents the establishment of stable cell–cell connections [69]. Quantitative analysis of shear stress on aggregate structure has reported that 6.5 dyn/cm$^2$ (0.65 Pa) is sufficient to remove the outer cells of an aggregate in suspension [76,77].

MSC aggregates are viscoelastic and are highly sensitive to mechanical force [78]. They exhibit solid-like properties when the stress is not large enough or over a short duration, but behave as liquid when the stress overcomes the sustainable threshold, inducing the cells to reorganize and change aggregate shape. In bioreactors, the 3D MSC aggregate may deform and reorganize as a result of external mechanical forces such as centrifugation and shear stress. The degree of deformation is determined by the ratio of external stress to aggregate stiffness, where stiffness is defined as the stress needed to achieve a certain deformation of a structure. Baraniak *et al.* reported that the Young's modulus for human MSC aggregates measured by a parallel-plate compression configuration is from $42.28 \pm 6.14$ Pa at day 2 to $62.4 \pm 5.58$ Pa at day 7, suggesting a reduction in deformation ability as the aggregates mature [18]. (Young's modulus, also known as the tensile modulus or elastic modulus, is a mechanical property of linear elastic solid materials. It measures the force (per unit area) that is needed to stretch (or compress) a material sample.) Based on the stiffness measurement, a 1% aggregate deformation is estimated in a bioreactor with shear stress in the range of 5–6.5 dyn/cm$^2$ (0.5–0.65 Pa). While the shear stress typically used in a bioreactor system has a limited impact on aggregate deformation, very high stress (15–30 dyn/cm$^2$, or 1.5–3.0 Pa) may reduce cell viability and induce apoptosis [79,80].

Bioreactor systems used in 3D MSC aggregate cultures are shown in Figure 8.3.

### 8.5.2 Spinner flasks

Spinner flasks are one of the simplest bioreactor systems with high scalability and have been used in MSC aggregate cultures. A spinner flask is a cylindrical glass container with an internal magnetic stirrer with two side-arms controlled by a stirring platform designed to agitate and aerate the medium to provide a homogeneous culture environment. One characteristic of the spinner flask is that higher agitation to achieve a well-mixed culture may generate turbulent flows, leading to a very high shear stress on MSC aggregates.

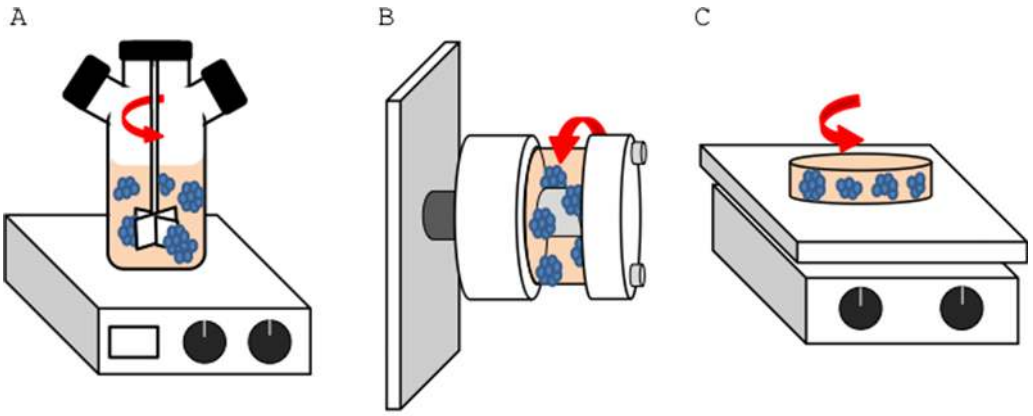
The flow rate and fluid shear in the spinner flask have been reported to be predominantly regulated by the impeller geometric shape and spatial position as well as the agitation speed, whereas the mass transfer coefficient increases as a power function of agitation speed under normal conditions [87]. Ismadi *et al.* reported that the mean shear stress in spinner flasks is a linear function of the impeller rotation speed and that the mean shear stress changes from 3 to 4.2 dyn/cm$^2$ (0.3 to 0.42 Pa) with two different types of impellers at a rotation speed 80 rpm [81]. In general, the highest shear stress level is typically at the end of the longest impeller [88] or at the base of the spinner flask, while the highest flow rate was observed in the meridional plane at the highest impeller position [81]. The profile of shear forces and the effect of hydrodynamics on MSC aggregates need to be considered when determining the aggregate size in the spinner flask bioreactor. Sen *et al.* used a formula proposed by Moreira to estimate the average diameter of neural stem cell aggregates in a spinner flask:

$$\text{Average diameter} = C\epsilon\alpha\nu/\beta$$

where $C$, $\alpha$, and $\beta$ are constants, $\epsilon$ is the power dissipated per unit mass, and $\nu$ is the medium kinematic viscosity [89]. This method can be applied to the dynamic culture of MSC aggregates. Given that Young's modulus of an MSC aggregate is around 520 dyn/cm$^2$ (52 Pa) and the shear stress in the spinner flask is around 3 dyn/cm$^2$ (0.3 Pa) with a rotation speed 80 rpm, the structural deformation is estimated to be less than 1%, suggesting that a spinner flask is a feasible method for MSC aggregate cultures [18,81].

### 8.5.3 Rotary wall vessel

The rotary wall vessel (RWV) system developed by NASA provides a microgravity environment for cell expansion and overcomes many limitations of the spinner flask bioreactor




| Spinner flask (A) | Rotary wall vessel (B) | | Rotary orbital shaker (C) |
|---|---|---|---|
| Rotation speed (rpm) | 30–70 | 11–25 | 45–65 |
| Shear stress (dyn/cm$^2$ (Pa)) | <5 (<0.5) | <1 (<0.1) | <1.5 (<0.15) |
| Aggregate size control | Yes | No | Yes |
| Reference | [27,49,81] | [27,82–85] | [18,61,73,86] |

**Figure 8.3** Comparison of three different types of bioreactors for aggregate formation and maintenance.

[90,91]. The structure of an RWV system is a horizontal cylinder with horizontal rotation and a silicon member for gas transfer. The laminar flow is driven by the outside horizontal vessel wall, which avoids the large shear stress associated with turbulent flow, thus providing a much more homogeneous shear stress field [92] and compensating for the gravity effect [91]. However, an RWV system retards the control of the MSC aggregate size, resulting in large variation of aggregate size because its low shear stress may increase the heterogeneous environment. The reported average shear stress in an RWV bioreactor is around 0.5–1.0 dyn/cm$^2$ (0.05–0.1 Pa), depending on rotation speed [82–85]. The rotation speed of the inner wall and the outer vessel in the same direction or in the opposite direction may not result in too much variation in velocity or stress profile [93]. Thus, the RWV bioreactor system provides a culture environment that improves mass transport with reduced mechanical stress on 3D cell aggregates.

### 8.5.4 Rotary orbital system

Some groups have used bacteriological-grade Petri dishes on a rotary orbital shaker to maintain MSC 3D aggregate cultures after aggregation by centrifugation [86]. It was reported that the rotary culture system increased the efficiency of the formation of embryoid bodies during embryonic stem cell culture and the uniformity in size distribution compared with that of static culture [94]. While direct formation of MSC 3D aggregates in the rotary orbital system has not been reported, increased mass transport under rotation helps maintain 3D aggregate viability and prevents their re-aggregation. Owing to the horizontal rotation without vertical circulation and noneven distribution of the fluid flow, the system has limited scalability. Computational analysis indicates that the maximum flow rate occurs in the peripheral region of the bottom of the well at a low rotation speed below 100 rpm, and at the center region of the bottom of the well at a high rotation speed such as 120 rpm. The maximum fluid shear occurs in the bottom of the well at around 1.5 dyn/cm$^2$ (0.15 Pa) and increases with the increase of the rotation speed [73].

### 8.5.5 Comparison of spinner flask and rotary wall vessel

Frith and colleagues developed dynamic 3D techniques for *in vitro* MSC culture using spinner flasks and an RWV bioreactor. They characterized these two methods for dynamic 3D MSC culture and compared the properties of these cultures with a conventional monolayer culture of MSCs. They showed that under optimal conditions MSCs formed compact cellular spheroids and remained viable in dynamic 3D culture. They demonstrated altered cell size and surface antigen expression together with enhanced osteogenic and adipogenic differentiation potential in the MSCs from the dynamic 3D conditions. By microarray analysis of monolayer and spinner flask MSCs they identified many differences in gene expression, including those confirming widespread changes to the cellular architecture and ECM. The upregulation of IL-24 in dynamic 3D cultures was shown to selectively impair the viability of prostate cancer cells cultured in medium conditioned by dynamic 3D MSCs [30]. However, anoikis, a form of programmed cell death that is induced by anchorage-dependent cells detaching from the surrounding ECM, was not entirely overcome [30].

### 8.5.6 Other systems

Other systems have been described, including the use of microcarrier beads, which provide a much greater surface area to which MSCs can adhere. Timmins *et al.* described the development of a closed process for bulk isolation of MSCs from human placenta and their subsequent cultivation on microcarriers in scalable single-use bioreactor systems. The authors estimated that a single placenta might produce over 7000 doses of therapeutic MSCs using a large-scale process [95].

The commercial company Terumo BCT sells its Quantum™ System, which is a functionally closed, automated, and scalable expansion method to grow preselected MSCs to yield clinically relevant doses in only 5 days.

## 8.6 Transplantation of three-dimensional mesenchymal stromal cell aggregates in preclinical animal models of disease

The functional enhancement induced by 3D aggregation of MSCs and the associated changes in structural and biophysical properties have given impetus to their application in preclinical studies in a variety of disease models. To date, suspension of single cells is the most common mode of cell delivery but suffers significant loss due to anoikis on detachment from the ECM, with a reduced ability to withstand an inflammatory *in vivo* environment [96]. Although trophism (support through trophic factors) is thought to be the main mechanism by which MSCs mediate their effects, increased survival and persistence of the transplanted cells at the wound site would likely be beneficial. To this end, transplantation of MSC aggregates or aggregate-derived cells appears to offer several advantages compared with transplantation of a single cell suspension. As discussed previously, 3D aggregation enhances the secretion of anti-inflammatory, anti-apoptotic, and immune modulating factors and increases their resistance to ischemic stress. The increased ECM in 3D human MSC aggregates protects them against damage induced by inflammatory cytokines at the wound site, increases cell adhesion, and prevents cell leakage. Because of their increased size, MSC aggregates are more readily visualized by *in vivo* imaging after their transplantation. While systemic administration of MSC aggregates is not feasible in some disease settings because of the possibility of vascular embolism, their transplantation has been actively pursued in several clinical models (Table 8.1).

### 8.6.1 Enhanced secretory properties of mesenchymal stromal cells aggregates

Multiple studies have shown that 3D MSC aggregation increases the expression of HIF and HIF-mediated genes, including VEGF, HGF, FGF-2, SDF-1α and CXCR4, and significantly enhances their proangiogenic and migratory properties. One application of the enhancement of angiogenesis by altering the secretion profile in aggregates is the salvage of the ischemic limb [49]. Hindlimb ischemia is a commonly used rodent model for the study of peripheral arterial disease and is induced by ligating the femoral artery and its branches through a skin incision. One day after the surgery, human ASDC aggregates were injected intramuscularly into the gracilis muscle of the medial thigh. Immunohistochemistry showed a three- to fivefold increase of angiogenic growth factors, including HGF, VEGF, and FGF-2, compared with 2D cultured cells. Immunofluorescent staining of murine von Willebrand Factor (vWF) and the quantification of the density of capillaries and arterioles indicated that transplantation of spheroid human ADSCs significantly enhanced the formation of capillaries and arterioles. The improvements were confirmed by histology. The mechanism of this enhanced therapeutic outcome is thought to be due to the combined effects of the increased angiogenic growth factors as well as higher survival of MSC aggregates in the ischemic damage sites compared with that of 2D cultured MSCs.

Similar findings for aggregate-mediated enhancement in angiogenesis have also been reported in animal models of oral mucositis [50]. Human gingival tissue-derived MSC aggregates or 2D cultured cells were injected intravenously into the areas of injury. Expression of VEGF and angiogenin in the MSC aggregates significantly enhanced the angiogenesis in the wound sites and promoted the restoration of weight loss due to decreased food intake because of the oral mucositis. (Angiogenin is a potent stimulator of new blood vessels through the process of angiogenesis.) In addition, the gingival MSC aggregates also had higher antioxidant property and exhibited higher resistance to oxidative stress at the wound site.

### 8.6.2 Immunomodulation by mesenchymal stromal cell aggregates

Another function of MSCs is downmodulation of excessive inflammatory responses by secretion of anti-inflammatory cytokines such as the IL-1 receptor antagonist, TSG-6, and $PGE_2$, which can be significantly enhanced by 3D aggregation. The murine model of zymosan-induced peritonitis has been used to evaluate the anti-inflammatory effects of MSC aggregates *in vivo* [51]. Aggregates and monolayer culture MSCs were injected intraperitoneally and the inflammatory exudates were collected by peritoneal lavage. There was a significant decrease of proinflammatory proteins in the lavage fluid in the MSC aggregate groups, including TNF-α, IL-1β, and CXCL2 (also known as macrophage inflammatory protein-1 (MIP-2)). Activity of serum plasmin, an inflammation-associated protease inhibited by TSG-6, was reduced to noninflammatory levels, suggesting that MSC aggregates are more effective in suppressing inflammation *in vivo* than MSCs cultured as monolayers.

### 8.6.3 Enhanced multilineage differentiation of three-dimensional mesenchymal stromal cells aggregates

Transplantation of 3D MSC aggregates enhances tissue repair and regeneration by better maintaining their multipotential lineage-specific differentiation compared with dissociated cells

**Table 8.1** Transplantation of MSC 3D aggregates in preclinical animal models.

| MSC source | Methods of aggregation and dissociation | Disease model | Method of delivery | Mechanisms of action | Ref. |
|---|---|---|---|---|---|
| Human gingival MSCs | 3 days of aggregation on ultra-low attachment plates | Oral mucositis | Intravenous infusion of dissociated aggregates | Increased stemness and trans-differentiation; increased secretion and migratory properties; higher resistance to oxidative stress | [50] |
| Human ADSCs | 4 days of hanging-drop culture | Parkinson's disease model | Intranigral[a] injection of dissociated human ADSCs | Enhanced endogenous neurogenesis; increased expression of prosurvival factors; stabilization of blood vessels | [97] |
| Human ADSCs | 3 days of spinner flask culture | Peripheral arterial disease (hindlimb ischemia) | Intramuscular injection of aggregates into the gracilis muscle[b] | Three- to fivefold increase in local HGF, VEGF, and FGF-2; two- to fivefold increase in host cells expressing α-actin, ICAM-1, VCAM-1 and PECAM-1 compared with dissociated cells | [49] |
| Human umbilical cord MSCs | 1 day hanging drop, then 2 days spinner flask culture | Peripheral arterial disease (hindlimb ischemia) | Intramuscular injection of aggregates into the gracilis muscle | Increased number of microvessels and smooth muscle α-actin compared with dissociated cells | [98] |
| Human bone marrow MSCs | 4 days of hanging drop culture | Peritonitis and inflammation | Intraperitoneal infusion of aggregates and aggregate-derived MSCs | Decrease of protein content including proinflammatory molecules and serum plasmin[c] | [51] |
| Human umbilical cord MSCs | 2 days of aggregation on ultra-low attachment plates | Myocardial infarction | Injection of aggregates into peri-infarct zones | Fourfold decrease in LV end-diastolic and systolic diameters; LV fractional shortening | [21] |
| Human ADSCs | 1 day of aggregation on maltose-binding protein-FGF2-coated surface | Myocardial infarction | Injection of aggregates into infarct border zones | Infarct size reduction and cardiomyocyte protection; threefold increase of human VEGF and angiogenesis; endothelial and smooth muscle cell differentiation | [99] |
| Human ADSCs | 5 days of microgravity bioreactor culture | Liver disease (acute liver failure) | Injection of aggregates into spleen | Improvement of liver function tests; suppressed cell necrosis; enhanced antiapoptosis | [100] |
| Human placental MSCs | 1 day hanging-drop culture | Ischemic stroke | Injection of aggregates into carotid arteries | Reduced cerebral infarct volume; restoration of neurologic functions; enhanced angiogenesis; neuronal differentiation | [101] |
| Rabbit bone marrow MSCs | 2 weeks of cell sheet culture | Bone non-unions and defects | Ectopic[d] and orthotopic[e] injection aggregates | Twofold increase in bone formation and higher bone mass compared with injection of single-cell suspension | [102] |
| Rabbit bone marrow MSCs | 2 weeks of cell sheet culture | Bone non-unions | Implantation of aggregates in dorsal pocket[f] | New bone and cartilage formation; enhanced compressive strength | [103] |
| Human synovial[g] MSCs | 3 days of hanging-drop culture | Cartilage defect | Osteochondral implantation of aggregates | Cartilage regeneration; defect was fully filled with cartilage matrix; border between cartilage and bone moved up | [104] |
| Rabbit articular cartilage and synovial MSCs | 2–3 days of low attachment dish with rotary shaker | Cartilage defect | Micropipette aspiration delivery of aggregates | Effective restoration of articular cartilage; higher *in vivo* ECM production | [105] |

[a] The substantia nigra is a brain structure located in the midbrain that plays an important role in reward, addiction, and movement. Parkinson's disease is characterized by the death of dopaminergic neurons in the substantia nigra.
[b] A slender superficial muscle in the thigh.
[c] Plasmin is an important enzyme present in blood that degrades many blood plasma proteins, including fibrin clots; the latter process is known as fibrinolysis.
[d] Located in an anatomical site other than the normal one for a given tissue or organ.
[e] Located in the normal anatomical site for a given tissue or organ.
[f] A triangular deepening on the radial dorsal aspect of the limb (in the hand in humans).
[g] The synovium or synovial membrane is the soft tissue found between the articular capsule (joint capsule) and the joint cavity of synovial joints.

in suspension. In the mouse hindlimb ischemia model, aggregates of human UCB-MSCs formed by the hanging-drop method were injected into the gracilis muscle and monitored for 4 weeks [98]. In addition to elevated values of VEGF and FGF-2, the number of vWF-positive microvessels, expression of NG2, and smooth muscle-α actin-positive vessels were increased in the spheroid transplantation group, revealing direct incorporation of human UCB-MSCs into blood vessels. NG2 (neural/glial antigen 2) is believed to play a role in functions such as cell adhesion, cell–cell and cell–ECM communication, cell migration, proliferation, as well as axonal growth, guidance, and regeneration. NG2-positive cells include oligodendrocyte progenitor cells and other progenitor cell populations, such as chondroblasts, myoblasts, and pericytes. Limb salvage of the human UCB-MSC spheroid group improved compared with that of MSC monolayer culture or control groups and was accompanied by decreased fibrosis in the ischemic region.

In the rat myocardial infarction model, human UCB-MSC aggregates prepared on ultralow attachment plates were injected into three different peri-infarct zones [21]. Results showed a fourfold decrease in left ventricular end-diastolic diameter, reduced systolic diameters, left ventricular fractional shortening, a reduction in cardiac fibrosis, and an increased density of capillaries. These results were thought to be due to increased engraftment in the aggregate group compared with that of the monolayer group. DiI-labeled MSC tracking revealed the expression of isolectin B4 and α-sarcomeric actin, indicating their differentiation into endothelial cells and cardiomyocytes respectively.

Similar findings of MSC differentiation into cells of the cardiac lineage were reported in another study that used MSC aggregates generated from maltose-binding protein-FGF-2-coated surfaces [99]. Cells in suspension and 3D MSC aggregates were injected into the infarct border zone at three locations after ligation of the left coronary artery induced an acute myocardial infarction. Histology and immunofluorescence staining of anti-human CD31 antibody (PECAM) and anti-human-α-smooth muscle actin antibody (hα-SMA) were used to verify endothelial cell and smooth muscle cell differentiation respectively. Densities of the $CD31^+$ and $\alpha\text{-}SMA^+$ cell in the 3D groups were higher than in the 2D group. The significant reduction in infarct size and better protection of cardiomyocytes were attributed to the direct differentiation of 3D MSC aggregates into smooth muscle cells and endothelial cells. Most of the differentiated cells in the 3D group were integrated into recipient blood vessels and contributed to neovascularization.

The enhanced multilineage potential of MSCs in 3D aggregates has also been demonstrated in other diseases, including liver disease and stroke. In a model of acute liver failure, MSC aggregates produced by a microgravity bioreactor were injected into the spleen [100]. A number of studies have shown that MSC aggregates have higher expression of pluripotent genes, such as *OCT-4*, *NANOG*, *SOX-2*, and *REX-1*, and a greater capacity to undergo lineage-specific differentiation compared to the MSCs cultured in monolayers. Expression of hepatic markers, including albumin, cytokeratin 18 (CK-18), and cytochrome P450 3A4 (CYP3A4), in human ADSC aggregates was increased in response to hepatocyte induction by conditioned medium. After transplantation into mice, levels of the hepatic enzymes aspartate aminotransferase and alanine aminotransferase were increased in mouse blood in those that received the MSC aggregates. Liver cell necrosis was also suppressed. While further studies are needed, these studies demonstrated that MSC 3D aggregates *may* have the capability to transdifferentiate into cells of the endoderm and ectoderm lineages, but this will need to be verified by specific *functional* tests.

Enhanced neurogenesis was observed in rats receiving MSC aggregates after an ischemic stroke due to occlusion of the middle cerebral artery [101]. *In vitro* expression of nestin and microtubule-associated protein 2 (MAP-2) in MSC aggregates could have been due to an enhancing effect on residual host nerve cells, or less likely due to MSC differentiation into nerve cells. It must be emphasized that there are very few, if any, convincing reports that MSCs can differentiate into functional cells of the ectoderm lineage. Nevertheless, in this model the MSC aggregate group showed a significant reduction in infarct volume, as well as an improved neurologic severity score and adhesive removal test. These results indicate recovery of neurologic functions in the brain after stroke. When tissue sections of the stroke brain were co-stained with antibodies against MAP-2 or the astrocyte-specific protein glial fibrillary acid protein (GFAP) and the human-specific nuclear protein (antibody M1281), there was a significant increase in co-expression of $MAP\text{-}2^+$ or $GFAP^+$ and $M1281^+$ cells in the spheroid MSC group.

### 8.6.4 Recapitulation of mesenchymal condensation and osteochondral differentiation in bone and cartilage regeneration

During embryonic development, condensation of previously dispersed cells is a pivotal stage in the development of mesenchymal tissues, including cartilage and bone. The 3D aggregates of MSCs have been shown to recapitulate mesenchymal condensation *in vitro* with enhanced chondrogenic and osteogenic potentials [106,107]. *In vivo* transplantation of scaffold-free MSC aggregates also enhanced mesenchymal condensation and osteochondral differentiation.

Rabbit bone marrow MSC aggregates have shown increased cell survival and enhanced bone healing through endochondral and intramembranous ossification compared with dissociated or 2D monolayer culture MSCs in a model of delayed bone fracture healing [102,103]. The mineralization mass was highly increased in the aggregate recipients. After 4 weeks, both bone (intramembranous ossification) and cartilage (endochondral ossification) formation were observed at the same time, with histomorphometry demonstrating approximately 33.3% bone and 17.5% cartilage of the cross-sectioned area. By 8 weeks, mineralized bone replaced some cartilage-like tissue, and bone

and cartilage occupied 56.5% and 7.1% of the total cross-sectional area respectively. Analysis of the fracture gap showed that the bone volume fraction of the aggregate group was significantly higher than that of the cell suspension or control groups. The expression of osteocalcin, collagen type I, osteopontin, and other osteogenic differentiation markers was also significantly higher in the MSC aggregate group. Moreover, when the mechanical properties of newly formed bone were measured, the compressive strength of the aggregate recipient group was greater than that of the 2D MSC group [103].

Transplantation of synovial MSC aggregates has been shown to enable their differentiation into chondrocytes and subsequently participate in the reparative process of osteochondral defects in the rabbit knee model [104]. MSC aggregates adhered promptly at the site of osteochondral defects without cell loss. Aggregation of human synovial MSCs also increased the expression of several chondrogenesis-related genes, including *BMP-2*. Transplanted aggregates of synovial MSCs directly differentiated into chondrocytes to participate in the reparative process. Four weeks after transplantation the osteochondral defect was mostly covered with a thick cartilaginous matrix. It was noted that increasing the number of MSC aggregates at the wound site did not result in improved healing. This may have been due to nutrition depletion and associated cell apoptosis or to reduced access to synovial fluid by the 3D aggregate at high density.

Undifferentiated MSCs can also aid chondrocyte differentiation in 3D aggregates of multiple cell types. In a study by Lee *et al.*, aggregate composites of chondrocytes and synovium-derived MSCs were transplanted into superficial osteochondral defects in a rabbit model [105]. Effective restoration of articular cartilage and *in vivo* ECM production were observed. The defects were covered with tissue that had a smooth surface, and the regenerated tissue was connected with adjacent normal cartilage when examined at 8 and 16 weeks after administration of the aggregates. No infiltration of inflammatory cells was found in the subchondral bone. Thus, MSCs may enhance musculoskeletal tissue repair and regeneration by supporting the host's parenchymal cells.

## References


1 Holtfreter, J. A study of the mechanics of gastrulation. *J Exp Zool* 1944;**95**(2):171–212.

2 Holtfreter, J. Neural induction in explants which have passed through a sublethal cytolysis. *Brain Res* 1947;**133**:329–339.

3 Moscona, A. The development in vitro of chimeric aggregates of dissociated embryonic chick and mouse cells. *Proc Natl Acad Sci U S A* 1957;**43**:184–194.

4 Sutherland, R.M. Cell and environmental interactions in tumor microregions: the multicell spehriods model. *Science* 1988;**240**: 177–184.

5 Su, G., Zhao, Y,. Wei, J,. *et al.* Direct conversion of fibroblasts into neural progenitor-like cells by forced growth into 3D spheres on low attachment surfaces. *Biomaterials* 2013;**34**(24):5897–5906.

6 Messina, E., De Angelis, L., Frati, G. *et al.* Isolation and expansion of adult cardiac stem cells from human and murine heart. *Circ Res* 2004;**95**(9):911–921.

7 Toma, J.G., Akhavan, M,. Fernandes, KJ,. *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001;**3**(9):778–784.

8 Matsuda, M., Imaoka, T., Vomachka, A.J., *et al.* Serotonin regulates mammary gland development via an autocrine–paracrine loop. *Dev Cell* 2004;**6**(2):193–203.

9 Seaberg, R.M., Smukler, S.R., Kieffer, T.J., *et al.* Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. *Nat Biotechnol* 2004;**22**(9): 1115–1124.

10 Singec, I., Knoth, R., Meyer, M.P., *et al.* Defining the actual sensitivity and specificity of the neurosphere assay in stem cell biology. *Nat Methods* 2006;**3**(10):801–806.

11 Korff, T. and Augustin, H.G. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol* 1998;**143**(5):1341–1352.

12 Bianco, P., Robey, P.G., and Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008;**2**(4): 313–319.

13 Serafini, M., Sacchetti, B., Pievani, A., *et al.* Establishment of bone marrow and hematopoietic niches *in vivo* by reversion of chondrocyte differentiation of human bone marrow stromal cells. *Stem Cell Res* 2014;**12**(3):659–672.

14 Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;**4**(5):267–274.

15 Hall, B.K. and Miyake, T. All for one and one for all: condensations and the initiation of skeletal development. *Bioessays*, 2000.**22**(2): 138–47.

16 Johnstone, B., Hering, T.M., Caplan, A.I., *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**(1):265–272.

17 Bhumiratana, S., Eton, R.E., Oungoulian, S.R., *et al.* Large, stratified, and mechanically functional human cartilage grown *in vitro* by mesenchymal condensation. *Proc Natl Acad Sci U S A* 2014; **111**(19):6940–6945.

18 Baraniak, P.R., Cooke, M.T., Saeed, R., *et al.* Stiffening of human mesenchymal stem cell spheroid microenvironments induced by incorporation of gelatin microparticles. *J Mech Behav Biomed Mater* 2012;**11**:63–71.

19 Wang, W., Itaka, K., Ohba, S., *et al.* 3D spheroid culture system on micropatterned substrates for improved differentiation efficiency of multipotent mesenchymal stem cells. *Biomaterials* 2009;**30** (14):2705–2715.

20 Foty, R.A. and Steinberg, M.S. The differential adhesion hypothesis: a direct evaluation. *Dev Biol* 2005;**278**(1):255–263.

21 Lee, E.J., Park, S.J., Kang, S.K., *et al.* Spherical bullet formation via E-cadherin promotes therapeutic potency of mesenchymal stem cells derived from human umbilical cord blood for myocardial infarction. *Mol Ther* 2012;**20**(7):1424–1433.

22 Schwartz, M.A. and D.W. DeSimone, Cell adhesion receptors in mechanotransduction. *Curr Opin Cell Biol* 2008;**20**(5): 551–556.

23 Hsu, S.H., Ho, T.T., Huang, N.C., *et al.* Substrate-dependent modulation of 3D spheroid morphology self-assembled in

mesenchymal stem cell–endothelial progenitor cell coculture. *Biomaterials* 2014;**35**(26):7295–7307.
24 Kapur, S.K., Wang, X., Shang, H., *et al.* Human adipose stem cells maintain proliferative, synthetic and multipotential properties when suspension cultured as self-assembling spheroids. *Biofabrication* 2012;**4**(2):025004
25 Lin, R.Z., Chou, L.F., Chien, C.C., and Chang, H.Y. Dynamic analysis of hepatoma spheroid formation: roles of E-cadherin and beta1-integrin. *Cell Tissue Res* 2006;**324**(3):411–422.
26 Caicedo-Carvajal, C.E., Shinbrot, T., and Foty, R.A. α5β1 integrin–fibronectin interactions specify liquid to solid phase transition of 3D cellular aggregates. *PLoS ONE*, 2010.**5**(7):e11830.
27 Frith, J.E., Thomson, B., and Genever, P.G. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods* 2010;**16**(4):735–749.
28 Potapova, I.A., Brink, P.R., Cohen, I.S., and Doronin, S.V. Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells. *J Biol Chem* 2008;**283**(19): 13100–13107.
29 Nesti, L.J., Caterson, E.J., Wang, M., *et al.* TGF-β1-stimulated osteoblasts require intracellular calcium signaling for enhanced α5 integrin expression. *Ann N Y Acad Sci* 2002;**961**: 178–182.
30 Frith, J.E., Thomson, B., and Genever, P.G. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. *Tissue Eng Part C Methods* 2009;**16**(4):735–749.
31 Ruiz, S.A. and Chen, C.S. Emergence of patterned stem cell differentiation within multicellular structures. *Stem Cells* 2008;**26** (11):2921–2927.
32 Rodriguez, J.P., González, M., Ríos, S., and Cambiazo, V. Cytoskeletal organization of human mesenchymal stem cells (MSC) changes during their osteogenic differentiation. *J Cell Biochem* 2004;**93**(4):721–731.
33 Dang, S.M., Gerecht-Nir, S., Chen, J., *et al.* Controlled, scalable embryonic stem cell differentiation culture. *Stem Cells* 2004;**22** (3):275–282.
34 Kinney, M.A., Hookway, T.A., Wang, Y., and McDevitt, T.C. Engineering three-dimensional stem cell morphogenesis for the development of tissue models and scalable regenerative therapeutics. *Ann Biomed Eng* 2014;**42**(2):352–367.
35 Shin, J.Y., Jeong, J.H., Han, J., *et al.* Transplantation of heterospheroids of islet cells and mesenchymal stem cells for effective angiogenesis and anti-apoptosis. *Tissue Eng Part A* 2015;**21** (5–6):1024–1035.
36 Chen, D.Y., Wei, H.J., Lin, W.W., *et al.* Intramuscular delivery of 3D aggregates of HUVECs and cbMSCs for cellular cardiomyoplasty in rats with myocardial infarction. *J Control Release* 2013;**172** (2):419–425.
37 Hoffecker, I.T. and Iwata, H. Manipulation of cell sorting within mesenchymal stromal cell-islet cell multicellular spheroids. *Tissue Eng Part A* 2014;**20**(11–12):1643–1653.
38 Jaiswal, N., Haynesworth, S.E., Caplan, A.I., and Bruder, S. Osteogenic differentiation of purified, culture-expanded human mesenchymal stem cells *in vitro*. *J Cell Biochem* 1997;**64**(2): 295–312.
39 Tang, J., Peng, R., and Ding, J. The regulation of stem cell differentiation by cell-cell contact on micropatterned material surfaces. *Biomaterials* 2010;**31**(9):2470–2476.
40 Denker, A.E., Haas, A.R., Nicoll, S.B., and Tuan, R.S. Chondrogenic differentiation of murine C3H10T1/2 multipotential mesenchymal cells: I. Stimulation by bone morphogenetic protein-2 in high-density micromass cultures. *Differentiation* 1999;**64**(2):67–76.
41 Shin, C.S., Lecanda, F., Sheikh, S., *et al.* Relative abundance of different cadherins defines differentiation of mesenchymal precursors into osteogenic, myogenic, or adipogenic pathways. *J Cell Biochem* 2000;**78**(4):566–577.
42 Yeh, H.Y., Liu, B.H., and Hsu, S.H. The calcium-dependent regulation of spheroid formation and cardiomyogenic differentiation for MSCs on chitosan membranes. *Biomaterials* 2012;**33** (35):8943–8954.
43 Hsu, S.H. and Huang, G.S. Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids. *Biomaterials* 2013;**34**(20):4725–4738.
44 Cheng, N.-C., Wang, S., and Young, T.-H. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. *Biomaterials* 2012;**33**:1748–1758.
45 Wang, Y.K., Yu. X., Cohen, D.M., *et al.* Bone morphogenetic protein-2-induced signaling and osteogenesis is regulated by cell shape, RhoA/ROCK, and cytoskeletal tension. *Stem Cells Dev* 2012;**21**(7):1176–1186.
46 Gao, L., McBeath, R., and Chen, C.S. Stem cell shape regulates a chondrogenic versus myogenic fate through Rac1 and N-cadherin. *Stem Cells* 2010;**28**(3):564–572.
47 Ichinose, S., Tagami, M., Muneta, T., and Sekiya, I. Morphological examination during in vitro cartilage formation by human mesenchymal stem cells. *Cell Tissue Res* 2005;**322**(2):217–226.
48 Van Winkle, A.P., Gates, I.D., and Kallos, M.S. Mass transfer limitations in embryoid bodies during human embryonic stem cell differentiation. *Cells Tissues Organs* 2012;**196**(1):34–47.
49 Bhang, S.H., Cho, S.W., La, W.G., *et al.* Angiogenesis in ischemic tissue produced by spheroid grafting of human adipose-derived stromal cells. *Biomaterials* 2011;**32**(11):2734–2747.
50 Zhang, Q., Nguyen, A.L., Shi, S., *et al.* Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis. *Stem Cells Dev* 2012;**21**(6):937–947.
51 Bartosh, T.J., Ylöstalo, J.H., Mohammadipoor, A., *et al.* Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc Natl Acad Sci U S A* 2010;**107**(31):13724–13729.
52 Skiles, M.L., Sahai, S., Rucker, L., and Blanchette, J.O. Use of culture geometry to control hypoxia-induced vascular endothelial growth factor secretion from adipose-derived stem cells: optimizing a cell-based approach to drive vascular growth. *Tissue Eng Part A* 2013;**19** (21–22):2330–2338.
53 Huang, C.C., Chen, D.Y., Wei, H.J., *et al.* Hypoxia-induced therapeutic neovascularization in a mouse model of an ischemic limb using cell aggregates composed of HUVECs and cbMSCs. *Biomaterials* 2013;**34**(37):9441–9450.
54 Kuschel, A., Simon, P., and Tug, S. Functional regulation of HIF-1α under normoxia – is there more than post-translational regulation? *J Cell Physiol* 2012;**227**(2):514–524.

55 Amos, P.J., Kapur, S.K., Stapor, P.C., *et al.* Human adipose-derived stromal cells accelerate diabetic wound healing: impact of cell formulation and delivery. *Tissue Eng Part A* 2010;**16**(5):1595–1606.

56 Covello, K.L., Kehler, J., Yu, H., *et al.* HIF-2α regulates Oct-4: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev*, 2006.**20**(5):557–70.

57 Choi, J., Kim, S., Jung, J., *et al.* Wnt5a-mediating neurogenesis of human adipose tissue-derived stem cells in a 3D microfluidic cell culture system. *Biomaterials* 2011;**32**(29):7013–7022.

58 Freyer, J.P. Rates of oxygen consumption for proliferating and quiescent cells isolated from multicellular tumor spheroids. *Adv Exp Med Biol* 1994;**345**:335–342.

59 Mueller-Klieser, W., Freyer, J.P., and Sutherland, R.M. Influence of glucose and oxygen supply conditions on the oxygenation of multicellular spheroids. *Br J Cancer* 1986;**53**(3):345–353.

60 Shim, J.H., Lee, T.R., and Shin, D.W. Novel *in vitro* culture condition improves the stemness of human dermal stem/progenitor cells. *Mol Cells* 2013;**36**(6):556–563.

61 Zimmermann, J.A. and McDevitt, T.C. Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion. *Cytotherapy* 2014;**16**(3):331–345.

62 Ylöstalo, J.H., Bartosh, T.J., Coble, K., and Prockop, D.J. Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells* 2012.**30**(10):2283–2296.

63 Bartosh, T.J., Ylöstalo, J.H., Bazhanov, N., *et al.* Dynamic compaction of human mesenchymal stem/precursor cells into spheres self-activates caspase-dependent IL1 signaling to enhance secretion of modulators of inflammation and immunity (PGE2, TSG6, and STC1). *Stem Cells* 2013;**31**(11):2443–2456.

64 Lee, R.H., Pulin, A.A., Seo, M.J., *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**(1):54–63.

65 Ylöstalo, J.H., Bartosh, T.J., Tiblow, A., and Prockop, D.J. Unique characteristics of human mesenchymal stromal/progenitor cells pre-activated in 3-dimensional cultures under different conditions. *Cytotherapy* 2014;**16**(11):1486–1500.

66 Niebruegge, S., Bauwens. C.L., Peerani, R., *et al.* Generation of human embryonic stem cell-derived mesoderm and cardiac cells using size-specified aggregates in an oxygen-controlled bioreactor. *Biotechnol Bioeng* 2009;**102**(2):493–507.

67 Subramanian, K., Park, Y., Verfaillie, C.M., and Hu, W.S. Scalable expansion of multipotent adult progenitor cells as three-dimensional cell aggregates. *Biotechnol Bioeng* 2011;**108**(2):364–375.

68 Zur Nieden, N.I., Cormier, J.T., Rancourt, D.E., Kallos, M.S. Embryonic stem cells remain highly pluripotent following long term expansion as aggregates in suspension bioreactors. *J Biotechnol* 2007;**129**(3):421–432.

69 Sart, S., Tsai, A.C., Li, Y., and Ma, T. Three-dimensional aggregates of mesenchymal stem cells: cellular mechanisms, biological properties, and applications. *Tissue Eng Part B Rev* 2014;**20**(5):365–380.

70 Kehoe, D.E., Jing, D., Lock, L.T., and Tzanakaki, E.S. Scalable stirred-suspension bioreactor culture of human pluripotent stem cells. *Tissue Eng Part A* 2009;**16**(2):405–421.

71 Ami, R.B., Barshtein, G., Zeltser, D., *et al.* Parameters of red blood cell aggregation as correlates of the inflammatory state. *Am J Physiol Heart Circ Physiol* 2001;**280**(5):H1982–H1988.

72 Moreira, J.L., Santana, P.C., Feliciano, A.S., *et al.* Effect of viscosity upon hydrodynamically controlled natural aggregates of animal cells grown in stirred vessels. *Biotechnol Prog* 1995;**11**(5):575–583.

73 Mogi, A., Takei, S., Shimizu, H., *et al.* Effects of fluid dynamic forces created by rotary orbital suspension culture on cardiomyogenic differentiation of human embryonic stem cells. *J Med Biol Eng* 2014;**34**(2):101–108.

74 Jossen, V., Pörtner, R., Kaiser, S.C., *et al.* Mass production of mesenchymal stem cells – impact of bioreactor design and flow conditions on proliferation and differentiation. In *Cells and Biomaterials in Regenerative Medicine*, D. Eberli (ed.). InTech, 2014.

75 Kolmogorov, A.N. The local structure of turbulence in incompressible viscous fluid for very large Reynolds numbers. *Dokl Akad Nauk SSSR* 1941;**30**:301–305.

76 Croughan, M.S., Sayre, E.S., and Wang, D.I. Viscous reduction of turbulent damage in animal cell culture. *Biotechnol Bioeng* 1989;**33**(7):862–872.

77 Fok, E.Y. and Zandstra, P.W. Shear-controlled single-step mouse embryonic stem cell expansion and embryoid body-based differentiation. *Stem Cells* 2005;**23**(9):1333–1342.

78 Tan, S.C., Pan, W.X., Ma, G., *et al.* Viscoelastic behaviour of human mesenchymal stem cells. *BMC Cell Biol* 2008;**9**:40.

79 Cherry, R.S. and Kwon, K.Y. Transient shear stresses on a suspension cell in turbulence. *Biotechnol Bioeng* 1990;**36**(6):563–571.

80 Liu, L., Yuan, W., and Wang, J. Mechanisms for osteogenic differentiation of human mesenchymal stem cells induced by fluid shear stress. *Biomech Model Mechanobiol* 2010;**9**(6):659–670.

81 Ismadi, M.-Z., Hourigan, K., and Fouras, A. Experimental characterisation of fluid mechanics in a spinner flask bioreactor. *Processes* 2014;**2**(4):753–772.

82 Tsao, Y., Goodwin, T.J., Wolf, D.A., and Spaulding, G.F. Responses of gravity level variations on the NASA/JSC bioreactor system. *Physiologist* 1992;**35**(1 Suppl):S49–S50.

83 Tzukert, K., Gorodestky, R., Avrahami, I., *et al.* A novel dynamic matrix detachment model reveals a shift from apoptosis to necrosis in melanoma cells. *Cancer Lett* 2008;**272**(2):345–354.

84 Unsworth, B.R. and Lelkes, P.I. Growing tissues in microgravity. *Nat Med* 1998;**4**(8):901–907.

85 Pei, M., Solchaga, L.A., Seidel, J., *et al.* Bioreactors mediate the effectiveness of tissue engineering scaffolds. *FASEB J* 2002;**16**(12):1691–1694.

86 Baraniak, P.R. and T.C. McDevitt, Scaffold-free culture of mesenchymal stem cell spheroids in suspension preserves multilineage potential. *Cell Tissue Res* 2012;**347**(3):701–711.

87 Kinney, M.A., Sargent, C.Y., and McDevitt, T.C. The multiparametric effects of hydrodynamic environments on stem cell culture. *Tissue Eng Part B Rev* 2011;**17**(4):249–262.

88 Liovic, P., Šutalo, I.D., Stewart, R., *et al.* Fluid flow and stresses on microcarriers in spinner flask bioreactors. In *Proceedings of the 9th International Conference on CFD in the Minerals and Process Industries*, Melbourne, December 10–12, C. B. Solnordal, P. Liovic, G. W. Delaney, and P. J. Witt (eds). CSIRO, 2012.

89 Sen, A., Kallos, M.S., and Behie, L.A. Expansion of mammalian neural stem cells in bioreactors: effect of power input and medium viscosity. *Develop Brain Res* 2002.**134**(1):103–113.

90 Freed, L.E., Langer, R., Martin, I., *et al.* Tissue engineering of cartilage in space. *Proc Natl Acad Sci U S A* 1997;**94**(25):13885–13890.

91 Gao, H., Ayyaswamy, P., and Ducheyne, P. Dynamics of a microcarrier particle in the simulated microgravity environment of a rotating-wall vessel. *Microgravity Sci Technol* 1996;**10**(3):154–165.

92 Liu, Y., Liu, T., Fan, X., *et al. Ex vivo* expansion of hematopoietic stem cells derived from umbilical cord blood in rotating wall vessel. *J Biotechnol* 2006;**124**(3):592–601.

93 Song, K., Wang, H., Zhang, B., *et al.* Numerical simulation of fluid field and *in vitro* three-dimensional fabrication of tissue-engineered bones in a rotating bioreactor and *in vivo* implantation for repairing segmental bone defects. *Cell Stress Chaperones* 2013;**18**(2):193–201.

94 Carpenedo, R.L., Sargent, C.Y., and McDevitt, T.C. Rotary suspension culture enhances the efficiency, yield, and homogeneity of embryoid body differentiation. *Stem Cells* 2007;**25**(9):2224–2234.

95 Timmins, N., Kiel, G., Heazlewood, C., *et al.* Closed system isolation and scalable expansion of human placental mesenchymal stem cells. *Biotechnol Bioeng* 2012;**109**:1817–1826.

96 Copland, I.B. and Galipeau, J. Death and inflammation following somatic cell transplantation. *Semin Immunopathol* 2011;**33**(6):535–550.

97 Schwerk, A., Altschüler, J., Roch, M., *et al.* Human adipose-derived mesenchymal stromal cells increase endogenous neurogenesis in the rat subventricular zone acutely after 6-hydroxydopamine lesioning. *Cytotherapy* 2015;**17**(14):199–214.

98 Bhang, S.H., Lee, S., Shin, J.Y., Lee, T.J., and Kim, B.S. Transplantation of cord blood mesenchymal stem cells as spheroids enhances vascularization. *Tissue Eng Part A* 2012;**18**(19–20):2138–2147.

99 Kim, J.H., Park, I.S., Park, Y., *et al.* Therapeutic angiogenesis of three-dimensionally cultured adipose-derived stem cells in rat infarcted hearts. *Cytotherapy* 2013;**15**(5):542–556.

100 Zhang, S., Liu, P., Chen, L., *et al.* The effects of spheroid formation of adipose-derived stem cells in a microgravity bioreactor on stemness properties and therapeutic potential. *Biomaterials* 2015;**41C**:15–25.

101 Guo, L., Ge, J., Zhou, Y., *et al.* Three-dimensional spheroid-cultured mesenchymal stem cells devoid of embolism attenuate brain stroke injury after intra-arterial injection. *Stem Cells Dev* 2014;**23**(9):978–989.

102 Ma, D.Y., Zhong, C., Yao, H., *et al.* Engineering injectable bone using bone marrow stromal cell aggregates. *Stem Cells Dev* 2011;**20**(6):989–999.

103 Ma, D., Ren, L., Liu, Y., *et al.* Engineering scaffold-free bone tissue using bone marrow stromal cell sheets. *J Orthop Res* 2010;**28**(5):697–702.

104 Suzuki, S., Muneta, T., Tsuji, K., *et al.* Properties and usefulness of aggregates of synovial mesenchymal stem cells as a source for cartilage regeneration. *Arthritis Res Ther* 2012;**14**(3):R136.

105 Lee, J.I., Sato, M., Kim, H.W., and Mochida, J. Transplantatation of scaffold-free spheroids composed of synovium-derived cells and chondrocytes for the treatment of cartilage defects of the knee. *Eur Cell Mater* 2011;**22**:275–290; discussion 290.

106 Ghone, N.V. and Grayson, W.L. Recapitulation of mesenchymal condensation enhances in vitro chondrogenesis of human mesenchymal stem cells. *J Cell Physiol* 2012;**227**(11):3701–3708.

107 Kale, S., Biermann, S., Edwards, C., *et al.* Three-dimensional cellular development is essential for *ex vivo* formation of human bone. *Nat Biotechnol* 2000;**18**(9):954–958.

CHAPTER 9

# Cell–cell signaling pathways that regulate mesenchymal stromal cell differentiation

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## 9.1 Introduction

The cell–cell signaling mechanisms controlling mesenchymal stromal cell (MSC) behavior are often examined using heterogeneous cultures that may contain clonal cells of varying potencies alongside other stromal populations. While this approach may obscure some ligand-induced effects, it may also reveal important interrelationships between different stromal cell types. In addition, MSCs will reside in a multicellular niche *in vivo*, where heterotypic cell–cell interactions will be highly influential. From this perspective, we discuss some of the key signaling pathways controlling MSC differentiation.

## 9.2 Mesenchymal stromal cell signaling is dependent on its type

The term MSC (usually referring to mesenchymal stromal cells, and rarely in the literature to true mesenchymal *stem* cells) is widely used to describe adherent stromal cells with the capacity to differentiate into the skeletal lineages of bone, fat, and cartilage. There is significant clinical interest in MSCs, but in order for these cells to fulfill their therapeutic potential it is important to gain a better understanding of the signaling mechanisms that control their proliferation and differentiation *in vivo*. However, discrepancies in reports from different research groups are not uncommon, and these inconsistencies may be a consequence of differences in the nature of the cell populations being studied. Early work in the MSC field isolated non-hematopoietic cells from bone marrow. They were characterized as a fibroblastic, plastic-adherent cell population, a fraction of which exhibited clonal expansion capacity and could generate osteoblasts, adipocytes, and chondrocytes on heterotopic implantation *in vivo* in the absence of exogenous differentiation stimuli [1,2]. Since that time, standardized methods to identify and isolate MSCs are still lacking due to the absence of a single, definitive marker for these cells. Current protocols often generate a heterogeneous population of cells that is not always consistently uniform between research groups. Furthermore, recent evidence suggests that subpopulations of MSCs exist *in vivo* and disparities in expansion protocols may promote the growth of different subtypes, which may account for the observed variability in the cells being studied. It has been demonstrated that cells isolated from bone marrow and other tissues as diverse as adipose tissue, synovium, and umbilical cord appear morphologically similar and undergo trilineage differentiation to osteogenic, adipogenic, and chondrogenic cells when exposed to differentiation stimuli *in vitro* [3–5].

The implication of this has been that MSCs isolated from anatomically varied sites are functionally equivalent. However, the *in vivo* MSC will be exposed to a tissue-specific environment and, it should be assumed, receive signaling inputs from the resident cells. It should also be noted that during embryonic development there is no single common precursor cell that can

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give rise to all the different skeletal tissue types, let alone a common precursor cell for the diverse range of tissues from which it is reported that multipotent MSCs can be isolated. Bone formation occurs prior to development of the marrow stroma [6], and different parts of the skeleton arise from different regions of the embryo. Facial bones are derived from the neural crest, while the axial skeleton is derived from paraxial mesoderm and the limb bones from lateral plate mesoderm [7]. The stromal compartment is then established from local progenitor cells in each tissue [6]. Identifying specific, preferably tissue-specific, developmental MSC markers is crucial to understanding the signaling mechanisms that drive their growth and differentiation. Most progress has been made with bone marrow-derived MSCs.

## 9.3 Identity of bone-marrow-derived mesenchymal stromal cells

In the absence of a unique marker, the International Society for Cellular Therapy (ISCT) in 2006 proposed minimal criteria for classifying cells as MSCs [8]. These included adherence to plastic, demonstration of multipotent differentiation potential *in vitro*, and expression of the cell surface markers CD105/endoglin, CD73/ecto-5′-nucleotidase, and CD90/Thy-1 by ≥95% of the population. It was also stated that the cells must be negative for CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA class II (≤2% of the population positive for these markers). The CD34 negativity of MSCs, however, is somewhat controversial, as several reports suggest that MSCs freshly isolated from tissues do indeed express CD34, but that expression is lost with culture *in vitro* [9]. However, since these surface proteins are also expressed by several other cell types, these criteria alone are insufficient to identify a homogeneous population of cells. Although several other cell surface proteins, such as CD13, CD29, and CD44 [10,11], are known to be characteristic of human MSCs, their expression is also not restricted to MSCs and, therefore, are of limited use in generating a pure population of cells.

Several other cell surface proteins expressed on MSCs have since been proposed to further enrich the population. The monoclonal Stro-1 antibody was one of the earliest antibodies used to enrich for MSCs in human bone marrow [12]. The subset of cells recognized by this antibody are capable of multilineage differentiation and include almost all fibroblast colony-forming unit (CFU-F) cells [12–14]. However, recent findings that Stro-1 is also expressed by the endothelium question its use as an MSC marker *in vivo* [15]. CD106/vascular cell adhesion molecule-1 is also expressed on human MSCs and has been used in combination with Stro-1 to isolate a more purified MSC population [16]. CD106 was found to be expressed by a subset of 1.4% of Stro-1-positive cells, and this population was highly enriched for CFU-Fs with multipotent mesodermal differentiation potential [16]. Other markers used to identify human MSCs have included: CD166 (activated leukocyte adhesion molecule) [17,18]; CD63, which is mainly associated with the membranes of intracellular vesicles and is also known as HOP-26 [19]; the α1 integrin subunit CD49α [20,21]; and stage-specific embryonic antigen-4 [22]. Recently, more specific MSC markers, such as CD271 (low-affinity nerve growth factor receptor) [23,24] and CD146 (melanoma cell adhesion molecule) [25] have also been shown to be expressed by bone-marrow-derived MSCs.

However, standard protocols used to isolate MSCs include expansion *in vitro*, and several reports suggest that an alteration in cell phenotype is associated with time in culture (see Chapter 7). Whereas expression of CD73, CD90, CD105, and CD166 have been shown to remain constant with increased passage [11,26], expression of CD106, CD146, Stro-1, and CD34 have all been reported to decrease with time in culture [9,12,27,28]. Furthermore, it has been suggested that MSCs directly isolated from bone marrow are in fact CD44-negative, and that expression of this antigen is an artefact of *in vitro* culture [29]. Reports demonstrating MSC marker expression *in situ*, as well as phenotypic studies of MSCs freshly isolated from bone marrow, have provided cell surface protein expression information without the problems associated with manipulation of the cells *in vitro*. CD271 was shown to be expressed in human bone marrow *in situ* by perivascular and bone-lining stromal cells [30,31]. CD146 expression was also demonstrated in MSCs in the subendothelial layer of sinusoids in human bone marrow [25]. Furthermore, it has been reported that a subset of CD271-positive MSCs are also positive for CD146 expression. At least two subpopulations of MSCs have been proposed: $CD271^+/CD146^+$ MSCs, which reside in perivascular locations; and $CD271^+/CD146^-$ MSCs, which exist endosteally [31].

A rare population of Nestin-positive ($Nestin^+$) mesenchymal cells isolated from mouse bone marrow exclusively contain CFU-F activity, are capable of self-renewal and expansion in serial transplantations, and also demonstrate multilineage differentiation, thus fulfilling the criteria of true (mesenchymal) *stem* cells [32]. Furthermore, these cells are located in close proximity to the vasculature and hematopoietic stem cells (HSCs), and have been shown to form an essential element of the HSC niche through expression of genes required for HSC maintenance, such as CXC chemokine ligand (CXCL) 12 and stem cell factor (SCF). These $Nestin^+$ MSCs appear very similar to CXCL12-abundant reticular (CAR) cells, identified surrounding sinusoidal endothelial cells in mouse bone marrow [33,34]. CAR cells reside in locations very similar to those described for $Nestin^+$ MSCs, mainly in central areas of mouse bone marrow, but also near the endosteum, and also promote HSC maintenance. $Nestin^+$ MSCs and CAR cells could potentially be two overlapping cell populations, although $Nestin^+$ MSCs appear to represent a rarer, more primitive cell type, being capable of multilineage differentiation, extensive self-renewal, and containing all CFU-F activity in the bone marrow (reviewed in [35]). Expression of the Leptin receptor (LepR) has also been used to identify cells with similar properties in mice [36,37]. $LepR^+$ perivascular cells express CXCL12 and SCF and are important components of the HSC

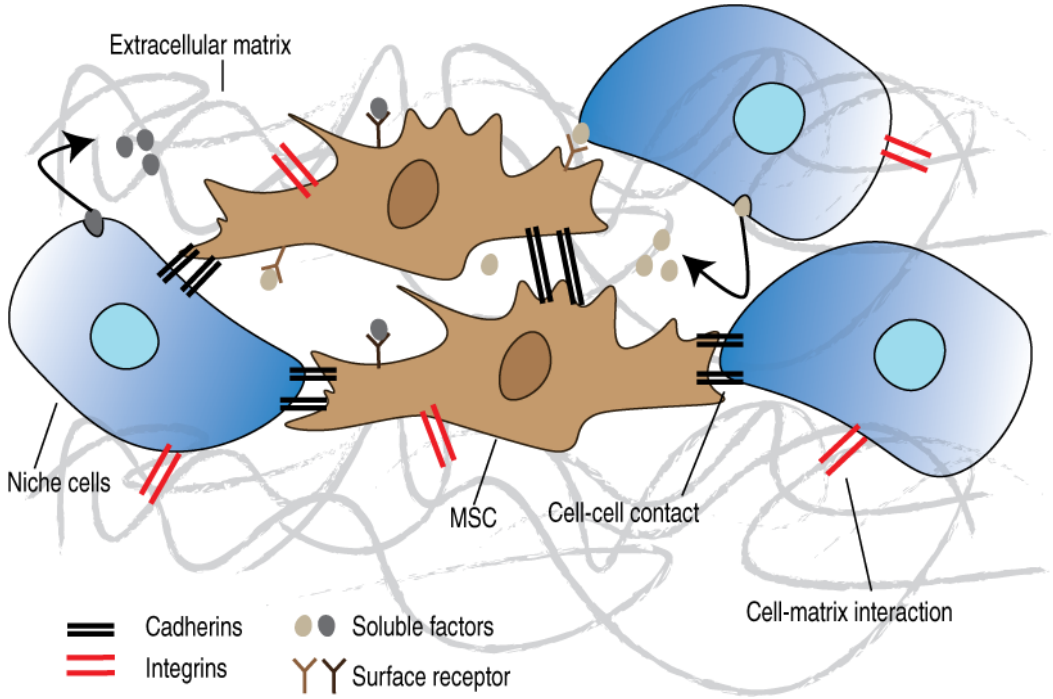



**Figure 9.1** The MSC niche.

niche. These LepR$^+$ MSCs have also been shown to account for most of the CFU-F activity in the bone marrow and are capable of multilineage differentiation [38]. A large overlap has been reported between LepR$^+$ cells and Nestin$^+$ populations in the bone marrow [39], though findings using lineage-tracing mice identified MSCs as LepR$^+$, SCF$^+$, CXCL12$^{high}$, and Nestin$^{low}$ [38].

An equivalent cell type has also recently been identified in humans. A subset of CD146$^+$ cells also express Nestin, as well as platelet-derived growth factor receptor α and CD51, and these cells were shown to be highly enriched in CFU-F activity, possess extensive self-renewal capacity, and express CXCL12 and SCF to support HSC maintenance [39].

Following the finding that MSCs are perivascular in both human [25] and in mouse [32] bone marrow, it has been proposed that MSCs are derived from pericytes [40–42]. Crisan *et al.* demonstrated that pericytes expressed a panel of MSC markers before culture, and that cultured pericytes display a phenotype similar to bone marrow MSCs, with proliferative capacity and multilineage differentiation potential. The common perivascular location *in vivo* and shared cell marker expression profiles of these two cell types has sometimes led to the suggestion that MSCs are pericytes [43].

Advances have been made in identifying authentic mesenchymal *stem* cells, most convincingly using mice models as described previously, which may not necessarily translate to human biology, and uncertainties persist. Functional markers, including cell surface receptors that can reveal more about MSC activity, are equivocal. Therefore, when examining the signaling inputs that regulate MSCs, it is often difficult to compare results across the scientific community where different antigens are used to identify MSCs or when the study relies on unsorted heterogeneous stromal cells where "average" effects will be reported. These comments are to provide context to the work reviewed here and are not intended to diminish the value of the published findings. One strategy gaining momentum has been to examine cell–cell signaling interactions in relation to the MSC niche (Figure 9.1).

## 9.4 Mesenchymal stromal cell signaling in the stem cell niche

Adult stem cells do not exist in isolation. They interact locally with their surrounding environment or niche, which is composed of a mixed community of stem cells and their differentiated progeny, niche-resident cells, and components of the extracellular matrix (ECM). The crosstalk between the stem cells and niche elements determines cell behavior and fate. Shear stress, oxygen gradient, neural input, and blood supply are also major determinants of stem cell function [44–46]. Progress has been made in defining niche composition and function for other adult stem cell types, including epithelial [47,48], intestinal [49], and neural [50] stem cells. The HSC niche [51] has received particular attention. In adult bone marrow, an endosteal HSC niche near the endosteal surface of the trabecular bone and a perivascular or

sinusoidal niche toward the center of the marrow have been described [52–54]. Further analyses indicated that HSCs preferentially occupied the perivascular niche, whereas lymphoid progenitors were endosteally located [36]. As discussed earlier, it is becoming clear that MSCs can be identified alongside HSCs in bone marrow. Mendez-Ferrer *et al.* revealed that Nestin$^+$ MSCs interact with HSCs at perivascular sites via CXCL12–CXCR4 signaling [32] and co-localize with sympathetic nerve fibers that can regulate HSC mobilization through circadian oscillations [55]. However, targeted depletion of CXCL12 in perivascular stromal cells was sufficient to deplete HSCs, whereas loss of CXCL12 from Nestin-*cre*-expressing cells had little effect on HSCs [36].

Detailed analysis of the *in vivo* MSC niche per se has been hampered by the lack of discriminating markers. Evidence that cells with MSC-like characteristics have been isolated from virtually every tissue type [56] would perhaps argue against the existence of a common MSC niche, with identical composition across all anatomical locations. However, evidence does suggest that MSCs are frequently associated with the vasculature and therefore reside in a perivascular niche [56], and several studies have determined the effects of MSC interactions with vascular endothelial cells (see Chapters 25 and 26). The intimate functional relationship between marrow angiogenesis and MSC differentiation has recently been demonstrated in the mouse [57]. Here, it was shown that Notch signaling in endothelial cells was required for appropriate blood vessel formation and osteo/chondrogenesis, with the Notch ligand Jagged 1 identified in perivascular stromal cells [57]. Using a three-dimensional co-culture model, it has been shown that endothelial cells significantly inhibited MSC proliferation, which may suggest a role in maintaining MSC quiescence or stemness. This study also demonstrated that, following exposure to differentiation stimuli, endothelial cells enhanced osteogenesis of MSCs at the expense of adipogenesis [58]. Related work has demonstrated enhanced osteogenesis in co-cultures of human MSCs and endothelial cells on polymeric microfiber scaffolds [59], whilst Xing *et al.* showed that endothelial cells enhanced *in vivo* bone formation when co-cultured with MSCs using poly(L-lactide-co-1,5-dioxepan-2-one) scaffolds [60]. Gene expression profiling has been used to help identify potential signaling mechanisms operating between MSCs and endothelial cells, using a direct-contact co-culture model [61], and identified the upregulation of genes associated with angiogenesis, such as von Willebrand factor, platelet/endothelial cell adhesion molecule-1 (PECAM1 or CD31), vascular endothelial growth factor, cadherin 5, angiopoietin-related protein 4, and CD34. Genes related to osteogenesis, such as alkaline phosphatase (ALP), FK506 binding protein 5, and bone morphogenetic proteins (BMPs), were also elevated. The authors concluded that the transforming growth factor-β (TGF-β)/BMP signaling pathway played an important role in the osteogenic induction of MSCs by endothelial cells [61], which has been noted elsewhere in studies that also highlighted the role of the Wnt signaling pathways [62]. These will be discussed in more detail later.

In addition, intercellular diffusion of signaling molecules through gap junctions appears important. It has been reported that communication through connexin 43 (CX43) contributes to osteogenic differentiation in two-dimensional co-cultures of endothelial cells and human MSCs [63,64] and the role of CX43 in osteogenesis has been demonstrated *in vitro* and *in vivo* [65,66]. Carrion *et al.* also reported that the interaction of MSCs with the laminin-rich vascular basement membrane deposited by endothelial cells via α6β1 integrin was essential for their perivascular localization [67]. Treating MSCs with an anti-α6 integrin antibody disrupted their adhesion to endotheial cell networks, preventing a perivascular association [67]. Matsubara *et al.* demonstrated that growth on a basement membrane-like ECM produced by primary endothelial cells enhanced MSC proliferative life-span and trilineage differentiation potential [68]. Other studies have focused on the regulation of niche ECM turnover by matrix metalloproteinases (MMPs). MSCs have been shown to protect their perivascular niche from high levels of endogenous MMP-2 through increased expression of tissue inhibitor of metalloproteinase (TIMP)-2 [69]. Even when exposed to pro-inflammatory cytokines and hypoxia, the matrix-protective property of MSCs was sustained by increased TIMP expression and thus augmented MMP-inhibition [69]. This highlights the importance of MSCs in regulating and being regulated by the structural organization of their local microenvironment, which includes stiffness and elasticity (see Chapters 15 and 17).

For example, varying matrix stiffness can exert a profound effect on the lineage specification and commitment of naive MSCs. MSCs grown on rigid matrices, resembling bone, promoted osteogenesis; softer matrices, resembling muscle, promoted myogenesis [70,71]. Additionally, MSCs are maintained in a quiescent state when cultured on soft polyacrylamide substrates to mimic the elasticity of the bone marrow niche [72]. There is evidence that mechanical stress may also influence MSC fate in the niche. The application of tensile mechanical strain to a collagen I substrate promoted osteogenesis of MSCs via the mitogen-acivated protein kinase signaling pathway [73]. Additionally, mechanical strain reduced the rate of MSC proliferation [74]. Likewise, bone-like ECM combined with fluid shear stress exposure synergistically enhanced the osteo-differentiation of three-dimensional cultures of MSCs even in the absence of the osteogenic supplement, dexamethasone [75], supported by findings that mechanical strain enhances osteogenesis but inhibits adipogenesis of MSCs [76].

It is clear that MSCs are influenced by a vast array of physical and biochemical factors. Two key signaling mechanisms that have interrelated regulatory effects on MSCs, either on undifferentiated cells in the niche or at different stages of differentiation and maturation, are the TGF-β/BMP and Wnt signaling pathways (Figure 9.2).

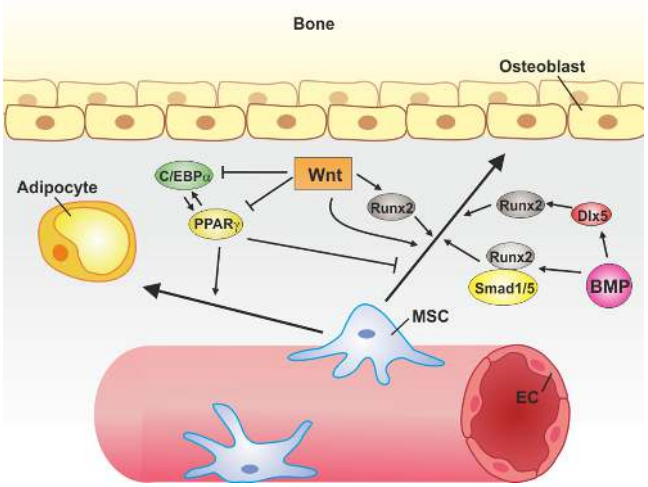



**Figure 9.2** Schematic representation of some of the signaling mechanisms that regulate MSC differentiation. MSCs are thought to occupy a perivascular niche where they can interact with endothelial cells (EC) and other vascular elements. Effects of Wnt and BMP on osteogenesis and adipogenesis through activation of key transcription factors are depicted. See Figure 9.3 for more detail of Wnt/BMP downstream signaling pathways. (See color plate section.)

## 9.5 Regulation of mesenchymal stromal cell differentiation by the TGF-β/BMP signaling pathway

The BMPs were initially identified as a group of proteins that induce endochondral bone formation [77] and are members of the TGF-β superfamily of signaling molecules. These secreted dimeric ligands bind to cell surface receptors with intracellular kinase activity. Ligand binding results in phosphorylation of specific receptor-regulated SMAD (R-SMAD) proteins. SMADs 2 and 3 are specific for TGF-β receptors, while SMADs 1, 5, and 8 are phosphorylated via BMP signaling. Following R-SMAD phosphorylation, an interaction complex is formed with Co-SMAD (SMAD4), and this complex is then able to translocate to the nucleus and regulate gene transcription [78]. Regulation of TGF-β/BMP signaling occurs via inhibitor SMAD (I-SMAD) proteins, which function in a negative feedback loop following upregulation by TGF-β ligands. I-SMADs can interfere with signal transduction by binding receptors and preventing R-SMAD phosphorylation, competing for complex formation between R-SMADs and SMAD4 and increasing receptor turnover [79] (Figure 9.3).

Signaling by TGF-β superfamily members regulates many aspects of embryonic development, including those related to specification of mesoderm and the differentiation of mesodermal progenitors into cells of skeletal lineages. During gastrulation, early mesendoderm is formed from epithelial cells during epithelial to mesenchymal transition (EMT). EMT is also a key part of the development of head structures from neuroepithelium-derived neural crest cells, which require TGF-βs and BMPs to generate craniofacial bone, cartilage, and connective tissue. TGF-$\beta3^{-/-}$ mice and those with a neural-crest-specific deletion of the BMP receptor Alk2 display cleft palate defects, demonstrating the requirement of TGF-β/BMP signaling in normal craniofacial development [79,80]. The importance of TGF-β/BMP signaling in mesoderm specification is also illustrated by work to derive

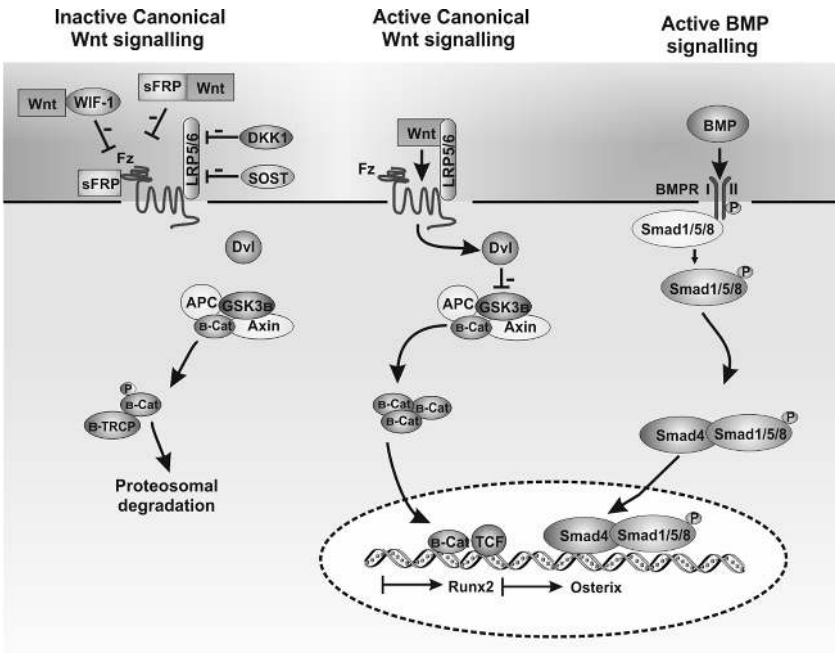



**Figure 9.3** Overview of the canonical Wnt and BMP signal transduction pathways. Wnt signaling is shown in the inactive (absence of Wnt signal) and active (following Wnt ligand binding) pathways. Crosstalk between Wnt and BMP intracellular signaling mechanisms will affect MSC responses to these regulatory cues.

induced pluripotent stem cells from fully differentiated cells by exogenous expression of pluripotent transcription factors (reprogramming). During the process of reprogramming, fully differentiated cells undergo dedifferentiation to a pluripotent state. Dedifferentiation can require mesenchymal to epithelial transition (MET), and it has been noted in a number of studies that inhibition of TGF-β signaling can improve the efficiency of this process [81,82]. It was also observed that addition of TGF-β1 or TGF-β2 acted as a barrier to reprogramming [82]. Taken together, these results demonstrate that TGF-β signaling acts to inhibit MET during reprogramming (dedifferentiation), which may be related to its role in driving the converse process of EMT during embryogenesis (differentiation).

BMP signaling also plays a role in embryonic limb and digit development. Targeted deletions of BMP2 and BMP4 in the limb mesenchyme resulted in failure to complete osteogenesis [83]. Digit defects, including polydactyly (extra digits) and syndactyly (interdigital webbing), were also observed when BMP2 and BMP4 were conditionally deleted in the apical ectodermal ridge [84] and the limb mesenchyme [83] of developing mouse embryos, demonstrating key roles for BMP signaling in limb skeletogenesis, including patterning and controlled cell death.

Along with its roles in embryonic development of the mesodermal lineage, BMP/TGF-β signaling also acts to regulate MSC differentiation *in vitro*. Recombinant BMP induces osteogenesis in preosteoblast cultures, while blocking BMP signaling inhibits osteogenesis [77]. BMPs act to induce the expression of the key osteogenic transcription factor Runx2. The induction of Runx2 by BMP signaling is mediated by another osteogenic transcription factor, Distal-less homolog (DLX5). DLX5 is specifically induced by BMP signaling, but not by other TGF-β superfamily members. It is expressed in response to BMP signaling and is upregulated prior to increased Runx2 expression. Robust BMP2 signaling cannot induce Runx2 expression in the absence of DLX5, indicating a key role for DLX5 in mediating BMP-induced Runx2 expression [85]. DLX5 also plays a vital role in the induction of ALP, which is known to be upregulated in response to BMP signaling. Runx2 has an additive effect on the DLX5-mediated expression of ALP, although DLX5 can induce ALP in the absence of Runx2, demonstrating that it acts independently of Runx2. Furthermore, the stimulation of ALP expression by BMP signaling is known to be abrogated in the absence of DLX5, demonstrating a critical role for DLX5 in BMP induction of ALP during osteogenesis [86]. The Runx2-independent induction of osteogenic markers by DLX5 is also observed for osterix (OSX), which is stimulated by BMP signaling via DLX5, even in Runx2-null cells. BMP signaling is unable to induce OSX expression in the absence of DLX5, again demonstrating the mediation of BMP signaling in osteogenesis via DLX5 [87]. Although DLX5 is clearly a critical regulator of BMP-induced osteogenesis, Runx2 also plays a vital role in transcriptional regulation of BMP-induced ostogenesis via interactions with SMAD 1 and SMAD 5. When BMP signaling is active, Runx2–SMAD complexes form and are targeted to specific nuclear matrix-associated sites where they regulate the expression of target genes [88]. The C-terminal domain of Runx2 is necessary for the induction of osteogenesis by BMP signaling, as it contains sequences for both SMAD interactions and nuclear matrix targeting required for the regulation of gene expression [89]. A triple mutation at positions 426–428 of the C-terminal domain of Runx2 is sufficient to disrupt Runx2–SMAD complex formation, prevent nuclear matrix targeting, and inhibit BMP-induced osteogenesis [90].

Unlike BMP signaling, TGF-β signaling acts to inhibit osteogenesis through the repression of pro-osteogenic BMP signals [91]. Although TGF-β1 acts to upregulate expression of the master osteogenic transcription factor Runx2, this does not result in osteogenic differentiation [85]. In fact, TGF-β acts through SMAD3 to inhibit osteogenesis by repressing the transcriptional activity of Runx2 [91]. Furthermore, specific inhibition of a TGF-β type I receptor kinase, or use of a TGF-β neutralizing antibody, is sufficient to enhance ALP and matrix mineralization in MSCs undergoing osteogenesis [92]. TGF-β also acts to inhibit adipogenic differentiation of MSCs. In contrast to its inhibitory activities in osteo- and adipogenesis, TGF-β, along with BMP signaling, induces chondrogenesis *in vivo* and *in vitro*. TGF-β stimulates the expression of chondrogenic genes in human MSCs *in vitro*, and also results in increased Alcian blue staining, indicative of glycosaminoglycan deposition. Interestingly, both its chondro-inductive and adipo-inhibitory roles are enhanced in the presence of lithium chloride (LiCl), a stimulator of Wnt signaling, suggesting TGF-β and Wnt signaling may work cooperatively to induce chondrogenesis and inhibit adipogenesis [93] (and see Chapter 14). Further crosstalk between the BMP and Sonic hedgehog/Indian hedgehog (SHH/IHH) signaling pathways is also necessary during chondrogenic differentiation. SHH signaling upregulates the expression of the NKX3.2 (also known as NK3 homeobox 2) protein, which acts as a transcriptional regulator, and BMP signaling then acts to maintain its expression [94]. NKX3.2 represses expression of factors that inhibit BMP-induced chondrogenesis. Interestingly, it also acts to repress the activity of Runx2 [95]. The combination of repression of anti-chondrogenic signals and the induction of the expression of the chondrogenic master regulator SOX9, which in turn can feedback and maintain expression of Nkx3.2 [96], allows the onset of chondrogenesis. The upregulation of SOX9 by BMP2 signaling also increases expression of the SOX9 downstream target collagen type 2, alpha 1 (Col2a1). BMP4 signaling can also induce SOX9 expression and the formation of ectopic cartilage in mouse mandibular extracts [97].

A common regulatory mechanism for BMP-induced differentiation is observed in both osteogenic and chondrogenic cultures. Despite similar upregulation of SOX9, ectopic cartilage was only observed when BMP4-soaked beads were implanted in proximal but not rostral regions of mouse mandibular extracts. The expression of the homeodomain transcription factor homeobox protein MSX-2 was also observed in mouse mandibular extracts, but at much higher levels in rostral than proximal regions. Furthermore, ectopic expression of Msx2 was sufficient

to inhibit BMP4-induced chondrogenesis, suggesting that Msx2 acts as a chondrogenic inhibitor [97]. Notably, Msx2 was also sufficient to inhibit BMP-induced osteogenic differentiation by competition with DLX5 for response element binding in the promoter of ALP. ALP was not expressed immediately after BMP signaling, when Msx2 levels were high and DLX5 expression was low. ALP induction occurred later, when Msx2 levels had dropped but DLX5 levels had risen, suggesting a threshold level regulation of osteogenic induction [86]. It was also possible that this occurred during chondrogenic induction in mouse mandibular extracts, thus providing a means of positional regulation of differentiation. It is interesting that Msx2 acts to regulate BMP-induced differentiation in both osteogenic and chondrogenic cultures. It has been demonstrated that Msx2 is involved in EMT in differentiating cultures of embryonic stem cells, and that BMP4 induces expression of Msx2 in these cultures [98]. Given that BMP signaling itself can induce Msx2 expression, this could suggest a mechanism of autoregulation in BMP-induced differentiation to osteogenic and chondrogenic lineages.

BMP signaling has clear roles in regulating MSC fate, but will operate alongside other regulatory pathways. Of fundamental importance is the cooperation of the BMP and Wnt signaling pathways in MSC regulation (Figure 9.3).

## 9.6 Regulation of mesenchymal stromal cell differentiation by the Wnt signaling pathway

Much research has focused on Wnt signaling since the discovery of mammalian Wnt1 [99], and such efforts have determined roles for this pathway in the regulation of many cellular activities, including cell proliferation, differentiation, and migration [100]. Nineteen mammalian Wnt genes have now been identified. These cysteine-rich secreted glycoproteins can signal either through the canonical β-catenin-dependent Wnt pathway or through noncanonical Wnt pathways that are independent of β-catenin activity. In the canonical pathway (Figure 9.3) the Wnt ligand binds to a Frizzled (Fz) receptor and a co-receptor, either low-density lipoprotein receptor-related protein (LRP) 5 or LRP6. This activates the intracellular protein Dishevelled (Dvl), which then inhibits a β-catenin destruction complex. This complex, involving axin, adenomatosis polyposis coli, and glycogen synthase kinase (GSK)-3β, causes β-catenin phosphorylation and its subsequent proteasomal degradation. Once stabilized by a canonical Wnt signal, β-catenin translocates to the nucleus and binds with members of the T-cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factor family to control expression of target genes. β-catenin-independent noncanonical Wnt signaling mechanisms include the Wnt/calcium pathway and the planar cell polarity (PCP) pathway. In the Wnt/calcium pathway the binding of specific Wnt ligands to Fz receptors activates calcium/calmodulin-dependent kinase II (CAMKII) and protein kinase C, whereas the PCP pathway activates Jun-N-terminal kinase to cause cytoskeletal rearrangements and regulate polarity within the tissue. Several antagonists ensure that Wnt signaling is tightly regulated. Secreted frizzled-related protein (SFRP) family members and Wnt inhibitory factor 1 inhibit the interaction between Wnt ligands and Fz receptors, whereas sclerostin and members of the Dickkopf (Dkk) family inhibit the Wnt co-receptors LRP5 and LRP6 (see Figure 9.3).

A vital role for canonical Wnt signaling in bone formation was first demonstrated when it was discovered that mutations in the Wnt co-receptor LRP5 led to skeletal defects. A gain-of-function mutation in LRP5, causing increased Wnt signaling, induced high bone mass in patients [101,102]. The same high bone mass phenotype was also seen in transgenic mice expressing a gain-of-function mutant LRP5 gene [103]. Conversely, a loss-of-function mutation in LRP5 causes osteoporosis-pseudoglioma (OPPG) syndrome in humans, resulting in reduced bone mass [104]. OPPG syndrome is a rare condition characterized by severe osteoporosis and eye abnormalities that lead to vision loss. The osteoporosis is usually recognized in early childhood. It is caused by a shortage of minerals, such as calcium, in bones (decreased bone mineral density), which makes the bones brittle and prone to fracture. Affected individuals often have multiple bone fractures, including in the vertebrae. Multiple fractures can cause collapse of the affected vertebrae, scoliosis (abnormal side-to-side curvature of the spine), short stature, and limb deformities. Most affected individuals have impaired vision at birth or by early infancy and are blind by young adulthood. Vision problems are usually caused by one of several eye conditions, grouped together as pseudoglioma, which affect the retina. Pseudogliomas are so named because, on examination, the conditions resemble a retinal glioma.

Furthermore, LRP5-deficient mice display a low bone mass phenotype reminiscent of OPPG syndrome in humans and caused by decreased proliferation and impaired function of osteoblasts [105]. Mutations in other components of the canonical Wnt signaling pathway, such as LRP6, sclerostin, SFRP4, and Dkk1, have now been similarly associated with abnormal bone mass, highlighting the importance of this pathway in bone formation [106–111].

Genetic mouse studies have revealed that one of the mechanisms through which canonical Wnt signaling increases bone mass is by stimulation of osteoblast differentiation, and evidence suggests that the canonical Wnt pathway is involved in numerous stages of the differentiation process. At early stages, conditional deletion of both LRP5 and 6 receptors, or β-catenin in mesenchymal progenitor cells, arrested differentiation into preosteoblasts, probably due to reduced expression of OSX [112–114]. At later stages, knockout of β-catenin in OSX-expressing osteoblasts inhibited their differentiation into mature osteoblasts [115], and in committed osteoblasts a lack of β-catenin caused deficiencies in maturation and mineralization [106]. Several studies using cell culture models have corroborated these results from mouse models and verified that

canonical Wnt signaling acts to stimulate osteogenic differentiation. In the mesenchymal cell line C3H10T1/2, expression of stabilized β-catenin, or the induction of β-catenin stabilization with either LiCl treatment or small molecule GSK-3β inhibitors, promoted osteoblast differentiation [116–118] (and see Chapter 13). More specifically, stimulation of canonical Wnt signaling by Wnt1, Wnt3a, and Wnt10b has been shown to stimulate osteoblastogenesis in *in vitro* and *in vivo* studies, while inhibition of Wnt signals using Dkk1 inhibits osteogenesis [117,119–121]. One mechanism through which canonical Wnt signaling may stimulate osteoblast differentiation is through upregulation of Runx2 [122]. SFRP1-null mice exhibit increased Wnt signaling levels and a high bone mass phenotype. Increased expression of TCF-1, Runx2, and the Runx2 target gene osteocalcin was also observed in these mutants [122]. Furthermore, a functional TCF regulatory element was identified in the promoter of Runx2, and elevated Runx2 expression was observed following co-expression of TCF1 and canonical Wnt proteins in osteoprogenitor cells, suggesting canonical Wnt signaling can directly activate Runx2 expression to promote osteoblast differentiation [122].

The discovery of the essential role of Wnt signaling in bone formation has provided targets for novel therapies to treat osteoporosis. Potentially, the downregulation of inhibitors of the canonical Wnt pathway may activate new bone formation to combat the low bone mass phenotype of this disease. Current targets for intervention include the Wnt antagonists Sclerostin and Dkk1 [123]. Dramatically increased bone formation, as well as bone mineral density, has been associated with downmodulation of each of these proteins. Accordingly, phase II clinical trials with an anti-Sclerostin antibody and preclinical trials with an anti-DKK1 antibody are underway [124,125].

Further evidence suggests that noncanonical Wnt signaling pathways are also involved in osteogenic differentiation. Downregulation of Wnt5a using neutralizing antibodies when culturing human MSCs inhibited their osteogenic differentiation [126]. Furthermore, $Wnt5a^{+/-}$ mice show reduced bone mass with decreased osteoblastogenesis [127], and deletion of Wnt5a specifically in osteoblast lineage cells also produces a low bone mass phenotype [128]. One study has determined that noncanonical Wnt signaling, stimulated by Wnt5a, can enhance osteoblast differentiation by upregulating LRP5 and 6 [129]. Signaling through another noncanonical Wnt family member, Wnt4, has also been reported to enhance bone regeneration by MSCs *in vivo* [130].

The canonical Wnt pathway is also involved in regulating adipogenic differentiation, with activation of canonical Wnt signaling acting to inhibit adipogenesis [131]. In preadipocytes, Wnt10b was found to inhibit the adipogenic transcription factors CCAAT-enhancer binding protein-α and peroxisome proliferator-activated receptor-γ (PPAR-γ) and arrest adipogenic differentiation, maintaining the cells in an undifferentiated state [132]. The inhibition of PPAR-γ is mediated by upregulation of the chicken ovalbumin upstream promoter transcription factor II by canonical Wnt10b, which in turn recruits the silencing mediator of retinoid and thyroid hormone receptors co-repressor complex [133]. This complex binds to the PPAR-γ gene and inhibits its expression by keeping the chromatin in a hypoacetylated state. Stimulation of Wnt signaling with Wnt1 in preadipocytes also causes inhibition of PPAR-γ expression and blocks adipogenic differentiation [134]. The inhibition of PPAR-γ by canonical Wnt signaling is reciprocal, as increases in PPAR-γ cause inhibition of β-catenin signaling [135]. Conversely, inhibition of Wnt signaling in preadipocyte cells using a dominant-negative TCF4 leads to increased adipocyte differentiation [132]. These and other studies have highlighted an important role for the canonical Wnt signaling pathway in controlling adipogenic differentiation in MSCs. An active canonical Wnt signal maintains the cells in an undifferentiated multipotent state until it is suppressed to allow adipogenesis. Canonical Wnt signaling evidently has an inverse effect on MSC differentiation along osteogenic and adipogenic lineages. Addition of LiCl to stimulate canonical Wnt signaling in MSCs causes adipogenic differentiation to be suppressed and osteogenic differentiation to be enhanced [136]. Similarly, through stimulation of canonical Wnt signaling, Wnt10b inhibits adipogenesis from preadipocytes, but promotes osteogenesis both *in vivo* and *in vitro* [132,137] and other Wnt family members, Wnt6 and Wnt10a, also show similar effects [138]. Furthermore, inhibition of Wnt signaling with DKK1 in precursor cells suppresses osteogenesis and enhances adipogenesis, demonstrating that Wnt antagonists also follow the same inverse pattern [139].

As well as the effects of canonical Wnt signaling on adipogenic differentiation, noncanonical Wnt signaling pathways also appear to be important regulators of adipogenesis. The noncanonical Wnt5a has been shown to suppress adipogenic differentiation of MSCs through suppression of PPAR-γ expression [127]. Interestingly, noncanonical Wnt signals also appear to show the same inverse effect on osteogenic and adipogenic differentiation of MSCs, as Wnt5a induces osteogenesis of MSCs, while also inhibiting adipogenesis [140–142]. Therefore, Wnt signaling mechanisms appear to promote osteogenic and suppress adipogenic MSC differentiation, through both canonical and noncanonical pathways.

Canonical Wnt signaling is also involved in the chondrogenic differentiation of MSCs. Ectopic canonical Wnt signaling in the developing limb bud of mice was found to promote ossification and inhibit chondrocyte formation [114]. Conversely, inhibition of canonical Wnt signaling led to ectopic chondrocyte differentiation and reduced osteogenesis. Overexpression of Wnt8c, 9a or β-catenin caused enhanced chondrocyte hypertrophy in chick upper sternal chondrocytes *in vitro* [143], again demonstrating a role for Wnt signaling in chondrogenesis. These effects are mediated, at least in part, through the LEF activation of Runx2, which may then increase Col10A1 expression [143]. These findings are consistent with *in vitro* studies of human MSCs in which enhanced

chondrogenesis was observed after inhibition of Wnt signaling with DKK-1 and SFRP-1 [144].

## 9.7 Conclusions

MSCs are intriguing cells that have attracted growing global research interest. The potential therapeutic value of these cells in regenerative medicine and immunomodulation applications sits somewhat awkwardly with their enigmatic identity, which has sometimes clouded full understanding of MSC biology. Here, we have focused on some of the signaling stimuli that MSCs receive to regulate their behavior, including effects on differentiation.

Of equal interest is a clearer comprehension of the MSC secretome and how MSCs are able to modify local cell activity by the release of bioactive ligands (see Chapters 15, 18, and 19). Consequently, there is likely to be significant and complex bidirectional signaling interplay within the MSC cellular microenvironment, which may be dependent on MSC subtype or tissue location.

## References


1 Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone-marrow osteogenic stem-cells – *in vitro* cultivation and transplantation in diffusion-chambers. *Cell Tissue Kinet* 1987;**20**(3):263–272.

2 Owen M, Friedenstein AJ. Stromal stem-cells – marrow-derived osteogenic precursors. *CIBA Found Symp* 1988;**136**:42–60.

3 Kern S, Eichler H, Stoeve J, *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**(5):1294–1301.

4 Lv FJ, Tuan RS, Cheung KMC, Leung VYL. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* 2014;**32**(6):1408–1419.

5 Segawa Y, Muneta T, Makino H, *et al.* Mesenchymal stem cells derived from synovium, meniscus, anterior cruciate ligament, and articular chondrocytes share similar gene expression profiles. *J Orthop Res* 2009;**27**(4):435–441.

6 Bianco P. "Mesenchymal" stem cells. *Annu Rev Cell Dev Biol* 2014;**30**:677–704.

7 Olsen BR, Reginato AM, Wang WF. Bone development. *Annu Rev Cell Dev Biol* 2000;**16**:191–220.

8 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

9 Lin CS, Ning H, Lin G, Lue TF. Is CD34 truly a negative marker for mesenchymal stromal cells? *Cytotherapy* 2012;**14**(10):1159–1163.

10 Deans RJ, Moseley AB. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000;**28**(8):875–884.

11 Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411):143–147.

12 Simmons PJ, Torok-Storb B. Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1. *Blood* 1991;**78**(1):55–62.

13 Dennis JE, Carbillet JP, Caplan AI, Charbord P. The STRO-$1^+$ marrow cell population is multipotential. *Cells Tissues Organs* 2002;**170**(2–3):73–82.

14 Gronthos S, Graves SE, Ohta S, Simmons PJ. The STRO-$1^+$ fraction of adult human bone marrow contains the osteogenic precursors. *Blood* 1994;**84**(12):4164–4173.

15 Ning H, Lin G, Lue TF, Lin CS. Mesenchymal stem cell marker Stro-1 is a 75 kD endothelial antigen. *Biochem Biophys Res Commun* 2011;**413**(2):353–357.

16 Gronthos S, Zannettino AC, Hay SJ, *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003;**116**(Pt 9):1827–1835.

17 Bruder SP, Horowitz MC, Mosca JD, Haynesworth SE. Monoclonal antibodies reactive with human osteogenic cell surface antigens. *Bone* 1997;**21**(3):225–235.

18 Bruder SP, Ricalton NS, Boynton RE, *et al.* Mesenchymal stem cell surface antigen SB-10 corresponds to activated leukocyte cell adhesion molecule and is involved in osteogenic differentiation. *J Bone Miner Res* 1998;**13**(4):655–663.

19 Joyner CJ, Bennett A, Triffitt JT. Identification and enrichment of human osteoprogenitor cells by using differentiation stage-specific monoclonal antibodies. *Bone* 1997;**21**(1):1–6.

20 Deschaseaux F, Charbord P. Human marrow stromal precursors are α1 integrin subunit-positive. *J Cell Physiol* 2000;**184**(3):319–325.

21 Stewart K, Monk P, Walsh S, *et al.* STRO-1, HOP-26 (CD63), CD49a and SB-10 (CD166) as markers of primitive human marrow stromal cells and their more differentiated progeny: a comparative investigation in vitro. *Cell Tissue Res* 2003;**313**(3):281–290.

22 Gang EJ, Bosnakovski D, Figueiredo CA, *et al.* SSEA-4 identifies mesenchymal stem cells from bone marrow. *Blood* 2007;**109**(4):1743–1751.

23 Jones EA, Kinsey SE, English A, *et al.* Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* 2002;**46**(12):3349–3360.

24 Quirici N, Soligo D, Bossolasco P, *et al.* Isolation of bone marrow mesenchymal stem cells by anti-nerve growth factor receptor antibodies. *Exp Hematol* 2002;**30**(7):783–791.

25 Sacchetti B, Funari A, Michienzi S, *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**(2):324–336.

26 Bellayr IH, Catalano JG, Lababidi S, *et al.* Gene markers of cellular aging in human multipotent stromal cells in culture. *Stem Cell Res Ther* 2014;**5**(2):59.

27 Halfon S, Abramov N, Grinblat B, Ginis I. Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev* 2011;**20**(1):53–66.

28 Jung EM, Kwon O, Kwon KS, *et al.* Evidences for correlation between the reduced VCAM-1 expression and hyaluronan synthesis during cellular senescence of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2011;**404**(1):463–469.

29 Qian H, Le Blanc K, Sigvardsson M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. *J Biol Chem* 2012;**287**(31):25795–25807.

30 Cattoretti G, Schiro R, Orazi A, *et al.* Bone marrow stroma in humans: anti-nerve growth factor receptor antibodies selectively stain reticular cells *in vivo* and *in vitro*. *Blood* 1993;**81**(7):1726–1738.

31 Tormin A, Li O, Brune JC, *et al.* CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* 2011;**117**(19):5067–5077.

32 Mendez-Ferrer S, Michurina TV, Ferraro F, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**(7308):829–834.

33 Omatsu Y, Sugiyama T, Kohara H, *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* 2010;**33**(3):387–399.

34 Sugiyama T, Kohara H, Noda M, Nagasawa T. Maintenance of the hematopoietic stem cell pool by CXCL12–CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006; **25**(6):977–988.

35 Ehninger A, Trumpp A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011;**208**(3):421–428.

36 Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* 2013; **495**(7440):231–235.

37 Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 2012;**481**(7382):457–462.

38 Zhou BO, Yue R, Murphy MM, *et al.* Leptin-receptor-expressing mesenchymal stromal cells represent the main source of bone formed by adult bone marrow. *Cell Stem Cell* 2014;**15**(2):154–168.

39 Pinho S, Lacombe J, Hanoun M, *et al.* PDGFRα and CD51 mark human Nestin$^{+}$ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med* 2013; **210**(7):1351–1367.

40 Armulik A, Genove G, Betsholtz C. Pericytes: developmental, physiological, and pathological perspectives, problems, and promises. *Dev Cell* 2011;**21**(2):193–215.

41 Bianco P, Cao X, Frenette PS, *et al.* The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;**19**(1):35–42.

42 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

43 Caplan AI. All MSCs are pericytes? *Cell Stem Cell* 2008; **3**(3):229–230.

44 Spiegel A, Kalinkovich A, Shivtiel S, *et al.* Stem cell regulation via dynamic interactions of the nervous and immune systems with the microenvironment. *Cell Stem Cell* 2008;**3**(5):484–492.

45 Ferraro F, Lo Celso C, Scadden D. Adult stem cells and their niches. In: Meshorer E, Plath K (eds), *Cell Biology of Stem Cells*. Springer-Verlag: Berlin, 2010; pp. 155–168.

46 Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nat Rev Mol Cell Biol* 2011;**12**(10):643–655.

47 Slack JMW. Stem cells in epithelial tissues. *Science* 2000;**287** (5457):1431–1433.

48 Blanpain C, Lowry WE, Geoghegan A, *et al.* Self-renewal, multipotency, and the existence of two cell populations within an epithelial stem cell niche. *Cell* 2004;**118**(5):635–648.

49 He XC, Zhang HW, Li LH. Cellular and molecular regulation of hematopoietic and intestinal stem cell behavior. In: Ourednik J, Ourednik V, Sakaguchi DS, Nilsen-Hamilton M (eds), *Stem Cell Biology: Development and Plasticity*. New York Academy of Sciences: New York, 2005; pp. 28–38.

50 Gage FH. Mammalian neural stem cells. *Science* 2000;**287** (5457):1433–1438.

51 Moore KA. Recent advances in defining the hematopoietic stem cell niche. *Curr Opin Hematol* 2004;**11**(2):107–111.

52 Ehninger A, Trumpp A. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011;**208**(3):421–428.

53 Mendelson A, Frenette PS. Hematopoietic stem cell niche maintenance during homeostasis and regeneration. *Nat Med* 2014; **20**(8):833–846.

54 Morrison SJ, Spradling AC. Stem cells and niches: mechanisms that promote stem cell maintenance throughout life. *Cell* 2008; **132**(4):598–611.

55 Mendez-Ferrer S, Lucas D, Battista M, Frenette PS. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 2008;**452**(7186):442–447.

56 Crisan M, Chen CW, Corselli M, *et al.* Perivascular multipotent progenitor cells in human organs. In: Kanz L, Weisel KC, Dick JE, Fibbe WE (eds), *Hematopoietic Stem Cells VII*. Wiley-Blackwell: Malden, NY, 2009; pp. 118–123.

57 Ramasamy SK, Kusumbe AP, Wang L, Adams RH. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. *Nature* 2014;**507**(7492):376–380.

58 Saleh FA, Whyte M, Ashton P, Genever PG. Regulation of mesenchymal stem cell activity by endothelial cells. *Stem Cells Dev* 2011;**20**(3):391–403.

59 Gershovich JG, Dahlin RL, Kasper FK, Mikos AG. Enhanced osteogenesis in cocultures with human mesenchymal stem cells and endothelial cells on polymeric microfiber scaffolds. *Tissue Eng Part A* 2013;**19**(23–24):2565–2576.

60 Xing Z, Xue Y, Danmark S, *et al.* Effect of endothelial cells on bone regeneration using poly(L-lactide-co-1,5-dioxepan-2-one) scaffolds. *J Biomed Mater Res Part A* 2011;**96A**(2):349–357.

61 Xue Y, Xing Z, Bolstad AI, *et al.* Co-culture of human bone marrow stromal cells with endothelial cells alters gene expression profiles. *Int J Artif Organs* 2013;**36**(9):650–662.

62 Saleh FA, Whyte M, Genever PG. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional *in vitro* model. *Eur Cell Mater* 2011;**22**:242–257; discussion 257.

63 Villars F, Guillotin B, Amedee T, *et al.* Effect of HUVEC on human osteoprogenitor cell differentiation needs heterotypic gap junction communication. *Am J Physiol Cell Physiol* 2002;**282**(4): C775–C785.

64 Guillotin B, Bareille R, Bourget C, *et al.* Interaction between human umbilical vein endothelial cells and human osteoprogenitors triggers pleiotropic effect that may support osteoblastic, function. *Bone* 2008;**42**(6):1080–1091.

65 Guerrero J, Catros S, Derkaoui SM, *et al.* Cell interactions between human progenitor-derived endothelial cells and human mesenchymal stem cells in a three-dimensional macroporous polysaccharide-based scaffold promote osteogenesis. *Acta Biomater* 2013;**9**(9):8200–8213.

66 Chaible LM, Sanches DS, Cogliati B, *et al.* Delayed osteoblastic differentiation and bone development in Cx43 knockout mice. *Toxicol Pathol* 2011;**39**(7):1046–1055.

67 Carrion B, Huang CP, Ghajar CM, *et al.* Recreating the perivascular niche *ex vivo* using a microfluidic approach. *Biotechnol Bioeng* 2010;**107**(6):1020–1028.

68 Matsubara T, Tsutsumi S, Pan H, *et al.* A new technique to expand human mesenchymal stem cells using basement membrane

extracellular matrix. *Biochem Biophys Res Commun* 2004;**313**(3):503–508.

69 Lozito TP, Tuan RS. Mesenchymal stem cells inhibit both endogenous and exogenous MMPs via secreted TIMPs. *J Cell Physiol* 2011;**226**(2):385–396.

70 Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126**(4):677–689.

71 Engler AJ, Sweeney HL, Discher DE, Schwarzbauer JE. Extracellular matrix elasticity directs stem cell differentiation. *J Musculoskelet Neuronal Interact* 2007;**7**(4):335.

72 Winer JP, Janmey PA, McCormick ME, Funaki M. Bone marrow-derived human mesenchymal stem cells become quiescent on soft substrates but remain responsive to chemical or mechanical stimuli. *Tissue Eng Part A* 2009;**15**(1):147–154.

73 Ward DF, Salasznyk RM, Klees RF, *et al.* Mechanical strain enhances extracellular matrix-induced gene focusing and promotes osteogenic differentiation of human mesenchymal stem cells through an extracellular-related kinase-dependent pathway. *Stem Cells Dev* 2007;**16**(3):467–479.

74 Kearney EM, Farrell E, Prendergast PJ, Campbell VA. Tensile strain as a regulator of mesenchymal stem cell osteogenesis. *Ann Biomed Eng* 2010;**38**(5):1767–1779.

75 Datta N, Pham QP, Sharma U, *et al.* In vitro generated extracellular matrix and fluid shear stress synergistically enhance 3D osteoblastic differentiation. *Proc Natl Acad Sci U S A* 2006;**103**(8):2488–2493.

76 Sen B, Xie ZH, Case N, *et al.* Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable beta-catenin signal. *Endocrinology* 2008;**149**(12):6065–6075.

77 Sampath TK, Maliakal JC, Hauschka PV, *et al.* Recombinant human osteogenic protein-1 (hOP-1) induces new bone-formation *in vivo* with a specific activity comparable with natural bovine osteogenic protein and stimulates osteoblast proliferation and differentiation *in vitro*. *J Biol Chem* 1992;**267**(28):20352–20362.

78 Roelen BA, Dijke P. Controlling mesenchymal stem cell differentiation by TGFβ family members. *J Orthop Sci* 2003;**8**(5):740–748.

79 Wan M, Cao X. BMP signaling in skeletal development. *Biochem Biophys Res Commun* 2005;**328**(3):651–657.

80 Derynck R, Akhurst RJ. Differentiation plasticity regulated by TGF-β family proteins in development and disease. *Nat Cell Biol* 2007;**9**(9):1000–1004.

81 Ichida JK, Blanchard J, Lam K, *et al.* A small-molecule inhibitor of Tgf-β signaling replaces *Sox2* in reprogramming by inducing *Nanog*. *Cell Stem Cell* 2009;**5**(5):491–503.

82 Maherali N, Hochedlinger K. Tgfβ signal inhibition cooperates in the induction of iPSCs and replaces Sox2 and cMyc. *Curr Biol* 2009;**19**(20):1718–23.

83 Bandyopadhyay A, Tsuji K, Cox K, *et al.* Genetic analysis of the roles of BMP2, BMP4, and BMP7 in limb patterning and skeletogenesis. *PLoS Genet* 2006;**2**(12):e216.

84 Maatouk DM, Choi KS, Bouldin CM, Harfe BD. In the limb AER Bmp2 and Bmp4 are required for dorsal–ventral patterning and interdigital cell death but not limb outgrowth. *Dev Biol* 2009;**327**(2):516–523.

85 Lee MH, Kim YJ, Kim HJ, *et al.* BMP-2-induced Runx2 expression is mediated by Dlx5, and TGF-β1 opposes the BMP-2-induced osteoblast differentiation by suppression of Dlx5 expression. *J Biol Chem* 2003;**278**(36):34387–34394.

86 Kim YJ, Lee MH, Wozney JM, *et al.* Bone morphogenetic protein-2-induced alkaline phosphatase expression is stimulated by Dlx5 and repressed by Msx2. *J Biol Chem* 2004;**279**(49):50773–50780.

87 Lee M-H, Kwon T-G, Park H-S, *et al.* BMP-2-induced Osterix expression is mediated by Dlx5 but is independent of Runx2. *Biochem Biophys Res Commun* 2003;**309**(3):689–694.

88 Zaidi SK, Sullivan AJ, van Wijnen AJ, *et al.* Integration of Runx and Smad regulatory signals at transcriptionally active subnuclear sites. *Proc Natl Acad Sci U S A* 2002;**99**(12):8048–8053.

89 Bae JS, Gutierrez S, Narla R, *et al.* Reconstitution of Runx2/Cbfa1-null cells identifies a requirement for BMP2 signaling through a Runx2 functional domain during osteoblast differentiation. *J Cell Biochem* 2007;**100**(2):434–449.

90 Javed A, Bae JS, Afzal F, *et al.* Structural coupling of Smad and Runx2 for execution of the BMP2 osteogenic signal. *J Biol Chem* 2008;**283**(13):8412–8422.

91 Alliston T, Choy L, Ducy P, *et al.* TGF-β-induced repression of CBFA1 by Smad3 decreases cbfa1 and osteocalcin expression and inhibits osteoblast differentiation. *EMBO J* 2001;**20**(9):2254–2272.

92 Maeda S, Hayashi M, Komiya S, *et al.* Endogenous TGF-β signaling suppresses maturation of osteoblastic mesenchymal cells. *EMBO J* 2004;**23**(3):552–563.

93 Zhou S, Eid K, Glowacki J. Cooperation between TGF-β and Wnt pathways during chondrocyte and adipocyte differentiation of human marrow stromal cells. *J Bone Miner Res* 2004;**19**(3):463–470.

94 Murtaugh LC, Zeng L, Chyung JH, Lassar AB. The chick transcriptional repressor Nkx3.2 acts downstream of Shh to promote BMP-dependent axial chondrogenesis. *Dev Cell* 2001;**1**(3):411–422.

95 Lengner CJ, Hassan MQ, Serra RW, *et al.* Nkx3.2-mediated repression of Runx2 promotes chondrogenic differentiation. *J Biol Chem* 2005;**280**(16):15872–15879.

96 Zeng L, Kempf H, Murtaugh LC, *et al.* Shh establishes an Nkx3.2/Sox9 autoregulatory loop that is maintained by BMP signals to induce somitic chondrogenesis. *Genes Dev* 2002;**16**(15):1990–2005.

97 Semba I, Nonaka K, Takahashi I, *et al.* Positionally-dependent chondrogenesis induced by BMP4 is co-regulated by Sox9 and Msx2. *Dev Dyn* 2000;**217**(4):401–414.

98 Richter A, Valdimarsdottir L, Hrafnkelsdottir HE, *et al.* BMP4 promotes EMT and mesodermal commitment in human embryonic stem cells via SLUG and MSX2. *Stem Cells* 2014;**32**(3):636–648.

99 Nusse R, Varmus HE. Many tumors induced by the mouse mammary tumor virus contain a provirus integrated in the same region of the host genome. *Cel.* 1982;**31**(1):99–109.

100 Nusse R, Varmus H. Three decades of Wnts: a personal perspective on how a scientific field developed. *EMBO J* 2012;**31**(12):2670–2684.

101 Boyden LM, Mao J, Belsky J, *et al.* High bone density due to a mutation in LDL-receptor-related protein 5. *New Engl J Med* 2002;**346**(20):1513–1521.

102 Little RD, Carulli JP, Del Mastro RG, *et al.* A mutation in the LDL receptor-related protein 5 gene results in the autosomal dominant high-bone-mass trait. *Am J Hum Genet* 2002;**70**(1):11–19.

103 Babij P, Zhao W, Small C, *et al.* High bone mass in mice expressing a mutant LRP5 gene. *J Bone Miner Res* 2003;**18**(6):960–974.

104 Gong Y, Slee RB, Fukai N, *et al.* LDL receptor-related protein 5 (LRP5) affects bone accrual and eye development. *Cell* 2001;**107**(4):513–523.

105 Kato M, Patel MS, Levasseur R, *et al.* Cbfa1-independent decrease in osteoblast proliferation, osteopenia, and persistent embryonic eye vascularization in mice deficient in Lrp5, a Wnt coreceptor. *J Cell Biol* 2002;**157**(2):303–314.

106 Holmen SL, Giambernardi TA, Zylstra CR, *et al.* Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. *J Bone Miner Res* 2004;**19**(12):2033–2040.

107 Kokubu C, Heinzmann U, Kokubu T, *et al.* Skeletal defects in ringelschwanz mutant mice reveal that Lrp6 is required for proper somitogenesis and osteogenesis. *Development* 2004;**131**(21):5469–5480.

108 Morvan F, Boulukos K, Clement-Lacroix P, *et al.* Deletion of a single allele of the *Dkk1* gene leads to an increase in bone formation and bone mass. *J Bone Miner Res* 2006;**21**(6):934–945.

109 Nakanishi R, Shimizu M, Mori M, *et al.* Secreted frizzled-related protein 4 is a negative regulator of peak BMD in SAMP6 mice. *J Bone Miner Res* 2006;**21**(11):1713–1721.

110 Balemans W, Ebeling M, Patel N, *et al.* Increased bone density in sclerosteosis is due to the deficiency of a novel secreted protein (SOST). *Human Mol Genet* 2001;**10**(5):537–43.

111 Li X, Ominsky MS, Niu QT, *et al.* Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner Res* 2008;**23**(6):860–869.

112 Joeng KS, Schumacher CA, Zylstra-Diegel CR, *et al.* Lrp5 and Lrp6 redundantly control skeletal development in the mouse embryo. *Dev Biol* 2011;**359**(2):222–229.

113 Hu H, Hilton MJ, Tu X, *et al.* Sequential roles of Hedgehog and Wnt signaling in osteoblast development. *Development* 2005;**132**(1):49–60.

114 Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* 2005;**8**(5):739–750.

115 Rodda SJ, McMahon AP. Distinct roles for Hedgehog and canonical Wnt signaling in specification, differentiation and maintenance of osteoblast progenitors. *Development* 2006;**133**(16):3231–3244.

116 Bain G, Muller T, Wang X, Papkoff J. Activated β-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction. *Biochem Biophys Res Commun* 2003;**301**(1):84–91.

117 Jackson A, Vayssiere B, Garcia T, *et al.* Gene array analysis of Wnt-regulated genes in C3H10T1/2 cells. *Bone* 2005;**36**(4):585–598.

118 Kulkarni NH, Onyia JE, Zeng Q, *et al.* Orally bioavailable GSK-3α/β dual inhibitor increases markers of cellular differentiation *in vitro* and bone mass *in vivo*. *J Bone Miner Res* 2006;**21**(6):910–920.

119 Bennett CN, Longo KA, Wright WS, *et al.* Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci U S A* 2005;**102**(9):3324–3329.

120 Rawadi G, Vayssiere B, Dunn F, *et al.* BMP-2 controls alkaline phosphatase expression and osteoblast mineralization by a Wnt autocrine loop. *J Bone Miner Res* 2003;**18**(10):1842–1853.

121 Gregory CA, Gunn WG, Reyes E, *et al.* How Wnt signaling affects bone repair by mesenchymal stem cells from the bone marrow. *Ann N Y Acad Sci* 2005;**1049**:97–106.

122 Gaur T, Lengner CJ, Hovhannisyan H, *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* 2005;**280**(39):33132–33140.

123 Gatti D, Viapiana O, Fracassi E, *et al.* Sclerostin and DKK1 in postmenopausal osteoporosis treated with denosumab. *J Bone Miner Res* 2012;**27**(11):2259–2263.

124 Padhi D, Jang G, Stouch B, *et al.* Single-dose, placebo-controlled, randomized study of AMG 785, a sclerostin monoclonal antibody. *J Bone Miner Res* 2011;**26**(1):19–26.

125 Papapoulos SE. Targeting sclerostin as potential treatment of osteoporosis. *Ann Rheum Dis* 2011;**70**(Suppl 1):i119–i122.

126 Guo J, Jin J, Cooper LF. Dissection of sets of genes that control the character of wnt5a-deficient mouse calvarial cells. *Bone* 2008;**43**(5):961–971.

127 Takada I, Mihara M, Suzawa M, *et al.* A histone lysine methyltransferase activated by non-canonical Wnt signalling suppresses PPAR-γ transactivation. *Nat Cell Biol* 2007;**9**(11):1273–1285.

128 Maeda K, Kobayashi Y, Udagawa N, *et al.* Wnt5a–Ror2 signaling between osteoblast-lineage cells and osteoclast precursors enhances osteoclastogenesis. *Nat Med* 2012;**18**(3):405–412.

129 Okamoto M, Udagawa N, Uehara S, *et al.* Noncanonical Wnt5a enhances Wnt/β-catenin signaling during osteoblastogenesis. *Sci Rep* 2014;**4**:4493.

130 Chang J, Sonoyama W, Wang Z, *et al.* Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. *J Biol Chem* 2007;**282**(42):30938–30948.

131 Laudes M. Role of WNT signalling in the determination of human mesenchymal stem cells into preadipocytes. *J Mol Endocrinol* 2011;**46**(2):R65–R72.

132 Ross SE, Hemati N, Longo KA, *et al.* Inhibition of adipogenesis by Wnt signaling. *Science* 2000;**289**(5481):950–953.

133 Okamura M, Kudo H, Wakabayashi K, *et al.* COUP-TFII acts downstream of Wnt/β-catenin signal to silence PPARγ gene expression and repress adipogenesis. *Proc Natl Acad Sci U S A* 2009;**106**(14):5819–5824.

134 Liu J, Farmer Sr. Regulating the balance between peroxisome proliferator-activated receptor γ and β-catenin signaling during adipogenesis. A glycogen synthase kinase 3β phosphorylation-defective mutant of β-catenin inhibits expression of a subset of adipogenic genes. *J Biol Chem* 2004;**279**(43):45020–45027.

135 Moldes M, Zuo Y, Morrison RF, *et al.* Peroxisome-proliferator-activated receptor γ suppresses Wnt/β-catenin signalling during adipogenesis. *Biochem J* 2003;**376**(Pt 3):607–613.

136 Li HX, Luo X, Liu RX, *et al.* Roles of Wnt/β-catenin signaling in adipogenic differentiation potential of adipose-derived mesenchymal stem cells. *Mol Cell Endocrinol* 2008;**291**(1–2):116–124.

137 Bennett CN, Ouyang H, Ma YL, *et al.* Wnt10b increases postnatal bone formation by enhancing osteoblast differentiation. *J Bone Miner Res* 2007;**22**(12):1924–1932.

138 Cawthorn WP, Bree AJ, Yao Y, *et al.* Wnt6, Wnt10a and Wnt10b inhibit adipogenesis and stimulate osteoblastogenesis through a β-catenin-dependent mechanism. *Bone* 2012;**50**(2):477–489.

139 Gustafson B, Eliasson B, Smith U. Thiazolidinediones increase the wingless-type MMTV integration site family (WNT) inhibitor Dickkopf-1 in adipocytes: a link with osteogenesis. *Diabetologia* 2010;**53**(3):536–540.

140 Arnsdorf EJ, Tummala P, Jacobs CR. Non-canonical Wnt signaling and N-cadherin related β-catenin signaling play a role in mechanically induced osteogenic cell fate. *PloS One* 2009;**4**(4):e5388.

141 Bilkovski R, Schulte DM, Oberhauser F, *et al.* Role of WNT-5a in the determination of human mesenchymal stem cells into preadipocytes. *J Biol Chem* 2010;**285**(9):6170–6178.

142 Santos A, Bakker AD, de Blieck-Hogervorst JM, Klein-Nulend J. WNT5A induces osteogenic differentiation of human adipose stem cells via rho-associated kinase ROCK. *Cytotherapy* 2010;**12**(7): 924–932.

143 Dong YF, Soung do Y, Schwarz EM, *et al.* Wnt induction of chondrocyte hypertrophy through the Runx2 transcription factor. *J Cell Physiol* 2006;**208**(1):77–86.

144 Im GI, Lee JM, Kim HJ. Wnt inhibitors enhance chondrogenesis of human mesenchymal stem cells in a long-term pellet culture. *Biotechnol Lett* 2011;**33**(5):1061–1068.

CHAPTER 10

# Regulation of mitochondrial transport in mesenchymal stromal cells

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## 10.1 Introduction

Mitochondrial donation is being envisioned as the "magic bullet" for a multitude of genetic disorders with deleterious mitochondrial DNA (mtDNA) mutations and diseases involving mitochondrial dysfunction. A number of methods have been described in the literature for detecting intercellular mitochondrial transfer. These methods include microinjection of mitochondria into recipient cells [1–4], incubation of recipient cells with mitocytoplasts (prepared from cells with microinjected mitochondria) or intact purified mitochondria [5,6], microvesicle-mediated transfer [7], and direct transfer through the formation of tunneling nanotubes (TNTs) between donor and recipient cells [8–11] and see Chapter 21. Of these, mitochondrial transfer through TNTs has been observed in many organisms and is considered to be evolutionarily conserved [12,13]. While it has been shown that a variety of cell types are capable of communicating through TNTs, mesenchymal stromal cells (MSCs) have caught the attention of the scientific and medical communities owing to their unique properties, such as their capacity to home to a site of inflammation (see Chapters 22–24), their contribution to tissue repair and regeneration (see Chapters 45–62), and their modulation of immune response (see Chapters 33 and 34). MSCs have some advantages over other cell choices, like embryonic stem cells and induced pluripotent stem cells (iPSCs): they are not the subject of ethical challenges, they have minimal or no risk of inducing tumor formation, and they are poorly immunogenic. Thus, MSCs from donors that are unrelated to and major histocompatibility complex (MHC)-unmatched with the recipient can be used without any need for immune suppression of the recipient. Although the clinical application of MSCs extends beyond being mitochondrial donors, mitochondrial transfer appears to be one of the critical mechanisms by which MSCs mediate their therapeutic effect. In this chapter we will explore this characteristic of MSCs, emphasizing the various regulatory mechanisms guiding mitochondrial transport. We will trace the evolution and shaping of the concept of intercellular mitochondrial transport, summarize the studies in which mitochondrial transfer from MSCs to recipient cells has been observed, discuss the regulatory mechanisms of mitochondrial transport, and postulate what lies ahead in the use of MSCs as therapeutic mitochondrial donors. We will demonstrate how MSCs donate mitochondria and rescue cellular bioenergetics. We will show that mitochondrial Rho GTPase 1 (Miro 1) is a critical regulator of MSC mitochondrial donor function, and we will show that enhancing the mitochondrial donor capacity of MSCs by overexpressing Miro-1 increases their therapeutic efficacy.

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## 10.2 Intercellular organelle transport

### 10.2.1 Intercellular communication

Cellular synchronization by intercellular communication is critical to a number of physiological and pathological processes, such as development, cell motility, embryogenesis, and tumorigenesis [14–18]. Information transfer *in vivo* can be achieved either by soluble mediators, microvesicles, or physical contact. Cell-to-cell communication can be divided into long-range, short-range, and close-range communications. The secretions of the body's endocrine system are the best examples of long-range intercellular communication. One example is the hypothalamus–pituitary–gonadal axis, whereby hormones secreted in the brain, such as follicle-stimulating hormone, induce the secretion of sex (or gonadal) steroids such as testosterone and estradiol, from the testicles and ovaries respectively, and which in turn interact with androgen or estrogen receptors respectively. Extracellular vesicles (e.g., microvesicles and exosomes) also participate in long-range communication through transfer of macromolecules (e.g., mRNA, micro RNAs, and proteins) and organelles such as mitochondria [19]. However, only microvesicles are capable of organelle transport.

Short-range communications are also mediated by soluble chemical mediators that diffuse into the extracellular environment, but their effect is limited to the cell releasing them (autocrine signaling) and/or to neighboring cells located close by. This is termed paracrine signaling, examples of which include fibroblast growth factor and transforming growth factor-β.

Close-range communication is mediated through formation of physical connections between neighboring cells (e.g., electrical synapses in the brain) [20]. The physical connections can be either noncytoplasmic or cytoplasmic. The noncytoplasmic physical contacts involve membranous extensions without cytoplasmic continuity; for example, cytonemes and filopodial extensions. These connections are firmly attached to the substratum and support the transport of their cargo along the outer side of the membranous extensions. Cytoplasmic bridges ensure intercellular cytoplasmic continuity and allow the exchange of ions and small molecules. Gap junctions represent an example of this. TNTs are a special form of these cytoplasmic bridges that were initially reported by Rustom *et al.* [11]. These are significantly different from the noncytoplasmic membrane extensions in both structural and functional aspects, as discussed later. TNTs enable cells to exchange organelles, and this property has created widespread interest in terms of the basic understanding of intercellular communication and its clinical translation. While various subcellular organelles can be exchanged through these structures, mitochondrial transport has gained particular focus in recent years. Knowledge of mitochondrial biology will quickly substantiate its application to cell-based therapeutic agents.

### 10.2.2 Mitochondrial biology

Mitochondria are double membrane cytoplasmic organelles found in all nucleated eukaryotic cells. These are the sites for the majority of the generation of cellular energy, producing >90% of the cellular energy through oxidative phosphorylation [21]. Besides energy production, mitochondria are also known to play a pivotal role in various other cellular processes, including inter-organelle communication, calcium signaling, amino acid and fat metabolism, cell proliferation, and apoptotic signaling [22]. Mammalian mitochondria are considered remnants of an ancient proteobacterium, obtained through endosymbiosis about 1.5 billion years ago [23]. These organelles have a compact (16 569 base pairs (bp)) circular DNA (mtDNA), which is thought to have transferred most of its genetic material to the nucleus during the course of evolution. mtDNA encodes only 37 genes, of which 13 are proteins coding for oxidative phosphorylation subunits, 22 are for transfer (t)RNAs, and 2 are ribosomal (r)RNAs associated with mitochondrial protein translation [24]. Another interesting characteristic of these organelles is cellular polyploidy [25]. The number of mitochondria in each somatic cell potentially varies between 200 and 1000 and is dependent on the energy requirements of the cell. For instance, skeletal muscle cells and neuronal cells contain the highest number of mitochondria. Each mitochondrion contains ~10–35 copies of mtDNA and carries a 10-fold increased risk of mutations in comparison with nuclear DNA due to three reasons: (a) the high oxidative environment of mitochondria due to free radical generation; (b) lack of DNA binding histones; and (c) ineffective repair mechanisms associated with mitochondria. The pathogenic mutations are not expressed until the mutation is accumulated in 60–90% of the total mtDNA of a somatic cell. This is called the threshold effect. Furthermore, single mitochondrion units are highly dynamic and constantly exchange their genetic material through mitochondrial fusion and fission, diluting the mutant mtDNA. This phenomenon is known as mitochondrial complementation [26,27]. Apart from mtDNA, mitochondrial proteins and lipids are also vulnerable to mitochondrial damage, which in turn may initiate a feed-forward process that results in additional damage and thus mitochondrial dysfunction.

Mitochondrial dysfunction has long been implicated in a wide array of inherited and acquired conditions, together known as mitochondrial cytopathies [21,22,28]. The concept that mitochondrial complementation can be imposed on the cells with mitochondrial damage by transferring healthy mitochondria is the basis for mitochondrial donation-based therapeutics.

### 10.2.3 Intercellular mitochondrial transport/ mitochondrial donation

Many different techniques for successful mitochondrial delivery, both *in vitro* and *in vivo*, to cells have been developed. The techniques include spontaneous engulfment of extracellular mitochondria by cells, cytoplast fusion (the cytoplast is the inner part of a cell without the cell wall, nucleus, or plasma membrane), microinjection of isolated mitochondria into specific cells, and transport of mitochondria either through the *de novo* formation

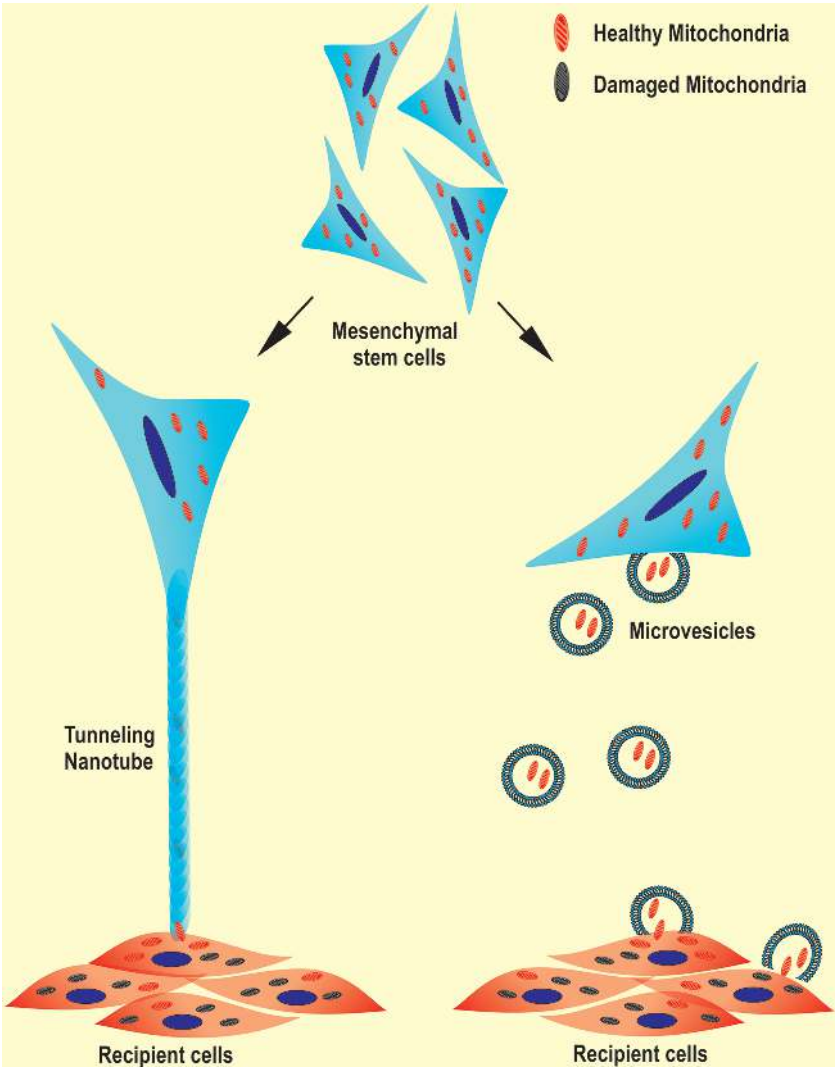



**Figure 10.1** Modes of intercellular transport of mitochondria from MSCs. Schematic diagram showing "Tunneling nanotube (TNT)" -mediated, and "Microvesicle"-packaged mitochondrial transfer.

of intercellular nanotubes (ICNs) or TNTs or through microvesicle-mediated delivery (Figure 10.1).

The mechanistic aspects and applicability of some of the important techniques are discussed in the following subsections.

#### 10.2.3.1 Microinjection of isolated mitochondria

This technique was pioneered by Tatum's group in *Neurospora crassa*, a species of fungus [1], and was later used by many other groups to generate cell lines with a host nucleus and foreign mitochondria known as transmitochondrial cell lines [2,4,26,29,30]. These cell lines have provided significant insights into mitochondrial genetics, mitochondrial complementation, and the role of nucleus–mitochondria compatibility in the survival of foreign mitochondria in recipient cells. Based on these studies, Pinkert *et al.* microinjected healthy mitochondria into fertilized murine oocytes and demonstrated the maintenance of the xenogenic (from a different species) mitochondria until the blastocyst stage of embryonic development (about 200–300 cells in humans) [3]. The results obtained from this study can be used along with assisted reproduction techniques to correct for maternal mtDNA mutations that lead to congenital mitochondrial diseases.

#### 10.2.3.2 Macropinocytosis of mitochondria

Clark and Shay demonstrated the spontaneous incorporation of isolated mitochondria stained with rhodamine$^{123}$ dye into recipient cells using fluorescence microscopy [2]. The spontaneous engulfment of isolated mitochondria was demonstrated in other studies [31–33], and Kitani and co-workers proposed that macropinocytosis is the underlying mechanism facilitating spontaneous mitochondrial uptake [5,6]. This mitochondrial transfer method has been called into question based on the negative results obtained in other studies [23,34]. Recently, cell-penetrating peptide-tagged mitochondria were engineered *in vitro* for efficient

cell penetration of mitochondria [34]. The therapeutic potential of this method was assessed in a rabbit model of myocardial infarction. The delivery of isolated autologous mitochondria through microinjection into the heart tissue was able to improve tissue adenosine triphosphate content and post-infarct cardiac function [33].

#### 10.2.3.3 Microvesicle-mediated mitochondrial delivery

Microvesicles are relatively large vesicles with a diameter of 100 nm to 1 μm and are shed from the plasma membrane of actively growing cells in a calcium- and calpain-dependent manner [19,35]. Calpain is a protein belonging to the family of calcium-dependent, nonlysosomal cysteine proteases (proteolytic enzymes) expressed ubiquitously in mammals and many other organisms. Microvesicles are capable of transferring macromolecules (mRNA, microRNA, and plasma membrane receptors). Microvesicles have recently been shown to be involved in the transfer of healthy mitochondria from MSCs to lung epithelial cells in a mouse model of acute lung injury *in vivo* [7]. This will be discussed further in subsequent sections.

#### 10.2.3.4 Tunneling nanotubes: highways for mitochondrial transport

TNTs are F-actin-based intercellular membrane channels facilitating the exchange of ions, plasma membrane components, vesicles, and organelles between remotely connected cells [9,36]. TNTs are also known to transport virus particles and pathogenic prion proteins [36]. These structures were first described by Rustom *et al.* in a rat phaeochromocytoma cell line (PC12) *in vitro* [11]. Following this pioneering observation, a number of reports demonstrated the formation of TNTs in a variety of cell types *in vitro*. TNTs connecting dendritic cells in the cornea of mice produced the first evidence of TNT formation *in vivo*, thus excluding the possibility of these structures being an *in vitro* artefact [37]. TNTs are involved in the transport of various subcellular organelles (mitochondria, lysosomes, Golgi complex, and endoplasmic reticulum), plasma membrane components, calcium ions, and electrical signals [9,36]. These functions performed by TNTs were found to be important for a wide range of physiological processes, including wound healing, cell migration, and neuronal communication [38]. Of all these functions, mitochondrial transport has gained a lot of attention in recent years owing to its application in restoring the bioenergetics of cells with mitochondrial damage. Subsequent sections explore the regulatory mechanisms in greater detail in the context of MSCs.

## 10.3 Mesenchymal stromal cells as potential mitochondrial donors

An MSC is a type of adult (or postnatal) progenitor cell found in the connective tissue of multiple organs. The therapeutic applications of these cells have evolved in line with knowledge of MSC biology [39]. Initially, these cells were evaluated for their tissue regeneration capabilities. The discovery of their role in cell proliferation, neovascularization, and immunomodulation further broadened the horizons of their therapeutic application for the treatment of inflammatory and immune-mediated diseases [40,41]. Prockop's group showed for the first time that mitochondria or mtDNA can move between cells, and thereby can rescue aerobic respiration in mammalian cells with nonfunctional mitochondria [23]. This discovery extended the potential clinical application of MSCs to a wide array of diseases associated with mitochondrial dysfunction [7,8,10,42–48].

Spees *et al.* co-cultured MSCs with mutant human lung alveolar epithelial cells (A549 cells) that are specifically depleted of mitochondrial genes and require a permissive medium additionally supplemented with pyruvate and uridine for growth. However, when co-cultured with human MSCs or fibroblasts, A549 cells showed rescue of proliferation and rate of growth even in restricted media. An analysis of the protein content in the revived population of A549 cells, derived from co-cultures of human MSCs and A549 ρ0 cells, revealed the presence of mitochondrial complex proteins such as COX-2 in the progeny A549 cells, which were absent in parental mutant A549 ρ0 cells. COX-2 is an enzyme responsible for inflammation and pain. Genetic screening of both the mitochondrial and nuclear DNA from the revived cells confirmed that the mitochondria in the revived cells were derived from either of the donor cells (human MSCs or fibroblasts). Cell fusion was excluded. The mitochondria that were received were metabolically active and capable of self-renewal. Notably, in the same study, an attempt to revive the A549 ρ0 cells with isolated mitochondria had failed, suggesting that specialized mechanisms were involved in mitochondrial donation by MSCs [23].

Further detailed evidence confirming the mitochondrial donor capacity of MSCs came in 2008 from the Zorov Laboratory exploring mitochondrial donation from MSCs to cardiomyocytes. In their co-cultures the transfer of mitochondria was via cytoplasmic bridge-like structures between MSCs and cardiomyocytes, and the movement of the mitochondria was exclusively unidirectional from MSCs to the cardiomyocytes [49]. An earlier study by Dimmeler's group had already shown that characteristic TNTs to facilitate transfer of mitochondria formed between co-cultured adult human endothelial progenitor cells and neonatal rat cardiomyocytes [50]. Based on these observations, Plotnikov and colleagues explored the possibility of transient cell-to-cell exchange of cytoplasm and organelles during co-culture of bone-marrow-derived MSCs and cultivated rat embryonic cardiac myocytes. In their study they observed the formation of TNTs in monocultures of MSCs and cardiomyocytes, which increased in number when the MSCs and cardiomyocytes were co-cultured. The study confirmed that intercellular TNT structures represented a mechanism for cytoplasmic continuity and bidirectional cytoplasmic flow. However, the mitochondrial transfer was exclusively unidirectional from the MSCs to the cardiomyocytes and could not thus be explained

by simple cytoplasm exchange. They reported vesicles with mitochondrial cristae structures traversing the TNTs, thereby concluding that "cytoskeleton filaments found within nanotubes may be an important element of directed vesicle-transporting machinery" [49]. Another important observation was the induced differentiation of MSCs after cytoplasmic transfer, leading to speculation that cytoplasmic exchange via TNTs could guide differentiation of MSCs into cardiomyocytes that would replace and repair injured myocardium. Similar findings were reestablished for co-cultivated human mesenchymal multipotent stromal cells and rat renal tubular cells [10]. Acquistapace *et al.* extended this concept further by finding transient partial cell fusion and mitochondrial transfer to differentiated mouse cardiomyocytes from human multipotent adipose-derived stromal (hMADS) cells [42]. A significant observation was that the terminally differentiated cardiomyocytes underwent somatic reprogramming to form cardiac progenitor-like cells when co-cultured with hMADS, rather than any evidence of hMADS differentiation. Thus, while mitochondrial transfer has been a consistent finding in multiple studies, its relative importance compared with cellular reprograming effects of cytoplasmic exchange was not clear.

Further confirmation of mitochondrial transfer by MSCs came from multiple laboratories around the world. Cho *et al.* confirmed that mtDNA-depleted ρ0-cells derived from human osteogenic sarcoma 143B cells could take in mitochondria from MSCs, with formation of cell–cell contacts and without cell fusion [44]. Dumler's group found that co-cultured MSCs and blood-vessel-derived vascular smooth muscle cells exchanged mitochondria bidirectionally, leading to MSC proliferation. This observation suggested that dual donor and recipient roles were also possible [51].

Together, these studies provided strong evidence for MSC-mediated mitochondrial transfer that appeared suitable for therapeutic purposes. Such therapeutic effects were further demonstrated in *in vivo* lung injury models by Bhattacharya's laboratory and our group [7,8].

Islam and colleagues provided the first *in vivo* evidence that bone marrow-derived MSCs formed connexin 43 (Cx43)-containing gap junctional channels with alveolar epithelium, releasing mitochondria-containing microvesicles that the epithelial cells engulfed [7]. In mice with septic peritonitis-induced acute lung injury, this attenuated alveolar injury and had marked survival benefits. In current work, we found that a Rho GTPase, Miro 1, which was known to regulate intracellular mitochondrial transport in neurons, also regulated intercellular mitochondrial transport from MSCs to lung epithelial cells (Figure 10.2).

Such mitochondrial transport from MSCs to airway epithelium was critical in attenuation of lung injury by rotenone or allergic inflammation. Rotenone is an odorless, colorless, crystalline ketonic chemical compound used as a broad-spectrum insecticide, piscicide, and pesticide.

An important role for directed TNT-mediated mitochondrial transport was suggested in our model since it was highly dependent on tumor necrosis factor, alpha-induced protein 2/M-Sec1, which regulates TNT formation. Together, these studies provided a mechanistic basis for MSC therapy in diseases characterized by mitochondrial dysfunction and inflammation. These include neurodegenerative diseases (amyotrophic lateral sclerosis, Alzheimer's disease) [52–54], respiratory diseases (chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis) [45,46,55–58], cardiovascular diseases (myocardial ischemia, myocardial infarction) [42,48,59,60], immune diseases (graft-versus-host disease, Crohn's disease) [40,61,62], and diabetes mellitus [63,64]. A number of phase I, II, and III clinical trials are in progress for the evaluation of the safety and therapeutic efficacy of MSCs in some of the diseases mentioned [65–67]. Important factors that would likely determine success include retention of MSCs at the site of injury, the dose of MSCs administered, and mitochondrial donor efficiency. Several strategies are being developed to improve the therapeutic efficiency of MSCs. In subsequent sections we explore the regulation of mitochondrial donation in more detail and describe strategies proposed to improve the mitochondrial donation capacity of MSCs.

### 10.3.1 Mechanism of intercellular mitochondrial transport regulation

Mitochondrial transport is now a well-studied characteristic of MSCs. However, mechanistic insight into its regulation is still in a nascent state. The various evidence and theories regarding the modes of transport (Figure 10.1) and the mechanisms, as understood so far, have been discussed. Passive diffusion is unlikely based on current understanding of membrane transport, and is not discussed further in view of lack of any strong evidence in support.

#### 10.3.1.1 Microvesicular export of mitochondria

Microvesicle-mediated transport as one of the underlying mechanisms for horizantal transfer of mitochondria between donor and target cells was initially suggested by Prockop's group [23]. However, this hypothesis did not initially receive much attention owing to lack of definitive evidence. Later, Islam *et al.* provided the first experimental evidence for microvesicle-mediated mitochondrial transport as one of the mechanisms for MSC-mediated rescue [7]. In a mouse model of acute lung injury they showed that calcium-dependent microvesicle shedding was strongly associated with the restoration of lung mitochondrial bioenergetics through mitochondrial transfer from bone-marrow-derived stromal cells (BMSCs) to alveolar epithelium. They also demonstrated the formation of closed-ended TNTs containing Cx43-mediated gap junctions *in vivo*. These TNTs were found to mediate the calcium communication essential for microvesicle shedding. Loss of mitochondrial transfer, either through expression of mutant Cx43 in BMSCs or inhibition of endocytosis of microvesicles through inhibition of dynamin GTPase, confirmed the role of microvesicles in the horizontal transfer of mitochondria *in vivo*. However, *in vitro* co-culture studies demonstrated spontaneous formation of open-ended TNTs supporting the transfer of mitochondria, and thus

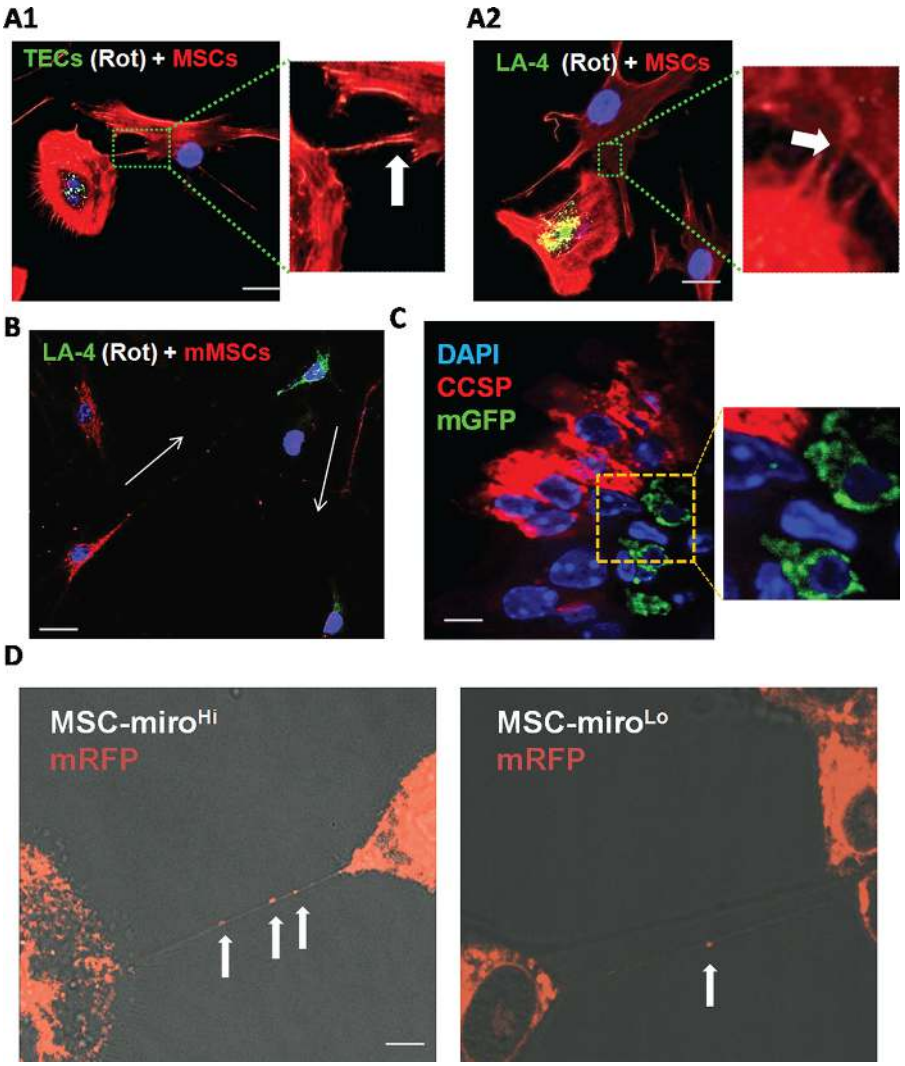



**Figure 10.2** TNT-mediated mitochondrial transfer from MSCs is regulated by Miro-1. (See color plate section.) **(A1 and A2)** Representative images showing TNTs between rotenone-treated epithelial cells (LA-4 or TECs) and MSCs. LA-4 cells or TECs were labeled with mGFP, while MSCs were unlabeled and co-cultured for 24 h, followed by fixation and staining with phalloidin to label TNTs. Insets show the zoomed-in portion of the images, while arrows indicate the TNT zoomed-in portions of the images. Scale bar: 10 μm **(B)** The image shows mitochondrial transfer from MSCs to stressed epithelial cells (LA-4 cells treated with rotenone). Prior to co-culture, MSCs were labeled with mitoRFP and the LA-4 were labeled with mitoGFP. Both cell populations were labeled with DAPI (blue), and the arrows indicate the unidirectional intercellular transfer of mitochondria from MSCs to LA-4 cells. Scale bar: 25 μm. **(C)** A representative image showing an allergically inflamed mouse lung tissue section labeled with the airway epithelial-cell-specific marker CCSP (red) and DAPI (blue) to label nuclei. This image was taken 24 h after mitoGFP-labeled MSCs were administered. Significant traces of green fluorescence can be seen in the lung. The enlarged section shows the mitoGFP-positive signals in lung epithelium. Scale bar: 30 μm. **(D)** The figure shows that mitochondrial movement is regulated by Miro-1.The left panel shows MSCs with overexpression of Miro-1 (cDNA treated), resulting in higher intercellular mitochondrial movement, in comparison with the right panel showing MSCs in which Miro-1 has been knocked down by shRNA. Arrows indicate the mitoRFP-labeled mitochondrial content moving inside the TNT as seen with phase-contrast microscopy. Further details can be found in [8]. Scale bar: 10 μm.

plausibly emphasized the co-occurrence of these two mechanisms. The following section focuses on the mechanisms underlying TNT-mediated mitochondrial transfer.

#### 10.3.1.2 Trafficking across intercellular nanotubular highways

In pioneering studies in nerve cells it was observed that mitochondria traversed the nerve cell body to energy-depleted sites all the way from the cyton (the cell body or soma of the neuron) to dendritic axons [68]. A set of proteins had been identified that constituted the intracellular mitochondrial transport machinery. This specific set of proteins were the group of mitochondrial Rho GTPases 1 and 2 (rhot1/Miro-1 and rhot2/Miro-2), which are anchored on to the outer membrane of mitochondria and are attached on to the kinesins or motor proteins via an adaptor protein termed Milton [69]. This helped the mitochondria to walk on the actin cytoskeletal flooring of cytoplasm. We hypothesized that the components

of intracellular mitochondrial transport could possibly also play a role in the intercellular mitochondrial transport across TNTs. MSCs were found to highly express the known critical components of intracellular mitochondrial transport machinery (Miro-1, Miro-2, Trak1, Trak2, KHC and Myo19). Knockdown of protein expression of Miro-1 with targeted shRNA in MSCs inhibited mitochondrial transfer from MSCs.

In naive MSCs, overexpression of approximately threefold upregulation in Miro-1 protein levels facilitated a significantly high amount of mitochondrial transport as well as efficient rescue and reversal of disease symptoms. The results were found to be similar when Miro-1 was overexpressed in human bronchial smooth muscle cells or fibroblasts used as mitochondrial donors *in vitro*. Taken together, these results confirmed that the enhancement effect on mitochondrial transfer was specific to modulation of Miro-1 expression.

The exact function of Miro-1 in MSC-mediated mitochondrial transfer is not known but is expected to be similar to its role in neurons. Kittler's group have shown that Miro-1 is involved in the calcium-dependent regulation of mitochondrial movement in neuronal cells [68]. Miro-1 protein has a transmembrane domain anchored into the mitochondrial outer membrane along with a couple of calcium sensor EF-hand domains flanked by two GTPase domains protruding into the cytoplasm. (The EF hand is a helix–loop–helix structural domain or motif found in a large family of calcium-binding proteins. The EF-hand motif contains a helix–loop–helix topology, much like the spread thumb and forefinger of the human hand, in which $Ca^{2+}$ ions are coordinated by ligands within the loop.)

The cytoplasmic domains of Miro-1 link the mitochondria to KIF5, a kinesin motor protein, which allows the movement of mitochondria on the microtubular network. Macaskill *et al.* demonstrated that the binding of Miro-1 to KIF5 is calcium-sensitive and is blocked at the intracellular sites with high calcium concentrations, leading to mitochondria mobility arrest [68]. This claim was substantiated by studies on EF-hand mutants of Miro-1 protein to prevent calcium binding, in which it was observed that these mutations had no effect on mitochondrial motility but calcium-dependent motility arrest was lost. So far they are the only known (mitochondrial) surface receptors that attach mitochondria to cytoskeletal structures via adaptors and motor proteins for their motility [70].

Recent studies probing the physiological significance of Miro-1 showed that the loss of Miro-1 which caused defects in mitochondrial motility was sufficient to potentiate neurological abnormalities in murine models [70]. Miro-1 has also been implicated as having a crucial role in the maintenance of mitochondrial turnover with PTEN-induced putative kinase 1, a mitochondrial serine/threonine-protein kinase, thought to protect cells from stress-induced mitochondrial dysfunction, as well as in PARKIN-mediated Miro degradation leading to arrested mitochondrial movement and capture autophagosomy (also known in this context as mitophagy – the selective degradation of mitochondria by autophagy; it often occurs in defective mitochondria following damage or stress) [71]. PARKIN is a protein that in humans is encoded by the *PARK2* gene. Its precise function is unknown, but the protein is a component of a multiprotein E3 ubiquitin ligase complex, which in turn is part of the ubiquitin-proteasome system that mediates the targeting of proteins for degradation.

We thus expect that the primary role of Miro-1 in MSCs is to regulate mitochondrial motility, and this can be harnessed for increased mitochondrial transfer. Effects of Miro-1 modulation on mitochondrial bioenergetics and turnover need to be better understood.

## 10.4 Strategies to improve mitochondrial delivery to target cells

Transplanted MSCs, after successful homing to the site of injury, have been demonstrated to exert their therapeutic effects in a number of ways. These include, first, promotion of tissue repair and regeneration either by engraftment in the recipient's tissue or by reprogramming the recipient's cells; second, by organelle donation through the formation of ICNs and/or microvesicle-mediated delivery of organelles and macromolecules; and, third, by paracrine signaling through the secretion of immunomodulatory cytokines and growth factors. Some lines of evidence suggest the interconnected nature of these mechanisms. As discussed previously, mitochondrial transfer through TNTs is also associated with cytoplasm exchange and reprogramming of donor or target cells. Similarly, transfer of mitochondria from vascular endothelial cells into MSCs has been shown to promote proliferation of the MSCs. Taken together, these reports suggest that the role of MSC-mediated mitochondrial transport has potential beyond the restoration of cellular bioenergetics. Therefore, it is logical to assume that potentiating mitochondrial donation capacity would enhance the therapeutic efficiency of MSCs. This is consistent with what we have seen in our study, where we enhanced mitochondrial transfer by overexpressing Miro-1 and found increased therapeutic efficacy. Miro-1-overexpressing-MSCs were more effective in restoring mitochondrial dysfunction and reversing pathological features (eosinophilic infiltration, airway hyperresponsiveness, mucin secretion, and subepithelial collagen deposition) in three independent mice models with experimentally induced allergic airway inflammation [8]. However, the enhanced therapeutic potential of Miro-1-overexpressed-MSCs may not warrant universal application since Miro-1-mediated mitochondrial mobility requires microtubules for regulation of the mitochondrial transport and is sensitive to high calcium in the local environment. To date, different types of TNTs with varied molecular compositions have been identified, and some of them did not contain microtubules. Further, Islam *et al.* showed that the movement of calcium through TNTs from damaged alveolar epithelial cells to the MSCs was a necessary event for

mitochondrial transfer [7]. Very high levels of calcium may not allow Miro-dependent intercellular mitochondrial transport through TNTs. However, microvesicle-mediated mitochondrial transport could still be active. Whether mitochondrial mobility stimulates or impedes microvesicle capture and release is not known. Strategies to stimulate TNT formation without impeding the function of Miro-1 may be suitable for increasing MSC efficacy, as may strategies to stimulate microvesicle-based transfer. These questions clearly require further exploration.

The relationship between MSC type and mitochondrial transfer also merits speculation. While little is known about the mitochondrial donation properties of various types of MSCs, there are clear differences in therapeutic efficacy. Accumulating data suggest that MSCs derived *ex vivo* from iPSCs (iPSC-MSCs) may have enhanced therapeutic potential compared with MSCs isolated from bone marrow or adipose tissue [61,72]. Taking leads from previous studies demonstrating the superior effect of iPSC-MSCs in attenuating limb ischemia and alleviating allergic airway inflammation, Li *et al.* have recently studied the role of iPSC-MSCs on cigarette-smoke-induced lung injury both *in vitro* and *in vivo* [58]. The results from their study confirmed the superior efficacy of iPSC-MSCs over normally derived MSCs in alleviating cigarette-smoke-induced lung damage. They attributed the observed enhanced potential to a high retention rate of iPSC-MSCs and high mitochondrial transport via TNTs. However, the role of paracrine effects and microvesicle-mediated transfer of macromolecules also require further investigation.

## 10.5 The road ahead

The use of MSCs as therapeutic agents for targeted mitochondrial delivery appears promising. This has particular promise in antiaging and regenerative medicine, as well as in the treatment of diseases characterized by mitochondrial dysfunction. While this is being explored in clinical trials, it is desirable that short- and intermediate-term metabolic consequences of MSC therapy be carefully studied. This is necessary to further refine the effectiveness of MSC therapy. Another area of interest is likely to be cancer, where delivery of mitochondria from MSCs may lead to cancer cell sensitization to toxins. We foresee that the incremental knowledge of MSC biology, supported by the rapidly developing tools to engineer MSCs, will lead to more MSC-based therapeutics emerging as the face of future medicine.

## References


1 E. G. Diacumakos, L. Garnjobst, and E. L. Tatum. A cytoplasmic character in *Neurospora crassa*: the role of nuclei and mitochondria. *J Cell Biol* 1965;**26**:427–443.

2 M. A. Clark and J. W. Shay. Mitochondrial transformation of mammalian cells. *Nature* 1971;**295**:605–607.

3 C. A. Pinkert, M. H. Irwin, L. W. Johnson, and R. J. Moffatt. Mitochondria transfer into mouse ova by microinjection. *Transgenic Res* 1997;**6**:379–383.

4 M. P. King and G. Attardi. Injection of mitochondria into human cells leads to a rapid replacement of the endogenous mitochondrial DNA. *Cell* 1988;**52**:811–819.

5 T. Kitani, D. Kami, T. Kawasaki, *et al.* Direct human mitochondrial transfer: a novel concept based on the endosymbiotic theory. *Transplant Proc* 2014;**46**:1233–1236.

6 T. Kitani, D. Kami, S. Matoba, and S. Gojo. Internalization of isolated functional mitochondria: involvement of macropinocytosis. *J Cell Mol Med* 2014;**18**:1694–1703.

7 M. N. Islam, S. R. Das, M. T. Emin, *et al.* Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 2013;**18**:759–765.

8 T. Ahmad, S. Mukherjee, B. Pattnaik, *et al.* Miro 1 regulates intercellular mitochondrial transport & enhances mesenchymal stromal cell rescue efficacy. *EMBO J* 2014;**33**:994–1010.

9 H. H. Gerdes, A. Rustom, and X. Wang. Tunneling nanotubes, an emerging intercellular communication route in development. *Mech Dev* 2013;**130**:381–387.

10 E. Y. Plotnikov, T. G. Khryapenkova, S. I. Galkina, *et al.* Cytoplasm and organelle transfer between mesenchymal multipotent stromal cells and renal tubular cells in co-culture. *Exp Cell Res* 2010;**316**:2447–2455.

11 A. Rustom, R. Saffrich, I. Markovic, *et al.* Nanotubular highways for intercellular organelle transport. *Science* 2004;**303**:1007–1010.

12 G. P. Dubey and S. Ben-Yehuda. Intercellular nanotubes mediate bacterial communication. *Cell* 2011;**144**:590–600.

13 W. J. Lucas, B. K. Ham, and J. Y. Kim. Plasmodesmata – bridging the gap between neighboring plant cells. *Trends Cell Biol* 2009;**19**:495–503.

14 E. Salas-Vidal and H. Lomeli. Imaging filopodia dynamics in the mouse blastocyst. *Dev Biol* 2004;**265**:75–89.

15 L. A. Elias, D. D. Wang, and A. R. Kriegstein. Gap junction adhesion is necessary for radial migration in the neocortex. *Nature* 2007;**448**:901–907.

16 C. Pyrgaki, P. Trainor, A. K. Hadjantonakis, and L. Niswander. Dynamic imaging of mammalian neural tube closure. *Dev Biol* 2010;**344**:941–947.

17 L. Caneparo, P. Pantazis, W. Dempsey, and S. E. Fraser. Intercellular bridges in vertebrate gastrulation. *PLoS ONE* 2011;**6**:e20230.

18 I. Antanavičiūtė, K. Rysevaitė, V. Liutkevičius, *et al.* Long-distance communication between laryngeal carcinoma cells. *PLoS ONE* 2014;**9**:e99196.

19 J. Ratajczak, M. Wysoczynski, F. Hayek, *et al.* Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 2006;**20**:1487–1495.

20 B. W. Connors and M. A. Long. Electrical synapses in the mammalian brain. *Annu Rev Neurosci* 2004;**27**:393–418.

21 S. R. Pieczenik and J. Neustadt. Mitochondrial dysfunction and molecular pathways of disease. *Exp Mol Pathol* 2007;**83**:84–92.

22 J. Nunnari and A. Suomalainen. Mitochondria: in sickness and in health. *Cell* 2012;**148**:1145–1159.

23 J. L. Spees, S. D. Olson, M. J. Whitney, and D. J. Prockop. Mitochondrial transfer between cells can rescue aerobic respiration. *Proc Natl Acad Sci U S A* 2006;**103**:1283–1288.

24 S. Anderson, A. T. Bankier, B. G. Barrell, *et al.* Sequence and organization of the human mitochondrial genome. *Nature* 1981;**290**:457–465.

25 K. L. Veltri, M. Espiritu, and G. Singh. Distinct genomic copy number in mitochondria of different mammalian organs. *J Cell Physiol* 1990;**143**:160–164.

26 K. Nakada, K. Inoue, T. Ono, *et al.* Inter-mitochondrial complementation: mitochondria-specific system preventing mice from expression of disease phenotypes by mutant mtDNA. *Nat Med* 2001;**7**:934–940.

27 A. Sato, K. Nakada, and J. I. Hayashi. Mitochondrial dynamics and aging: mitochondrial interaction preventing individuals from expression of respiratory deficiency caused by mutant mtDNA. *Biochim Biophys Acta* 2006;**1763**:473–481.

28 H. A. Tuppen, E. L. Blakely, D. M. Turnbull, and R. W. Taylor. Mitochondrial DNA mutations and human disease. *Biochim Biophys Acta* 2010;**1797**:113–128.

29 C. L. Bunn, D. C. Wallace, and J. M. Eisenstadt. Cytoplasmic inheritance of chloramphenicol resistance in mouse tissue culture cells. *Proc Natl Acad Sci U S A* 1974;**71**:1681–1685.

30 C. M. Croce. Loss of mouse chromosomes in somatic cell hybrids between HT-1080 human fibrosarcoma cells and mouse peritoneal macrophages. *Proc Natl Acad Sci U S A* 1976;**73**:3248–3252.

31 R. L. Elliott, X. P. Jiang, and J. F. Head. Mitochondria organelle transplantation: introduction of normal epithelial mitochondria into human cancer cells inhibits proliferation and increases drug sensitivity. *Breast Cancer Res Treat* 2012;**136**:347–354.

32 J. D. McCully, D. B. Cowan, C. A. Pacak, *et al.* Injection of isolated mitochondria during early repurfusion for cardioprotection. *Am J Physiol Heart Circ Physiol* 2009;**296**:H94–H105.

33 A. Masuzawa, K. M. Black, C. A. Pacak, *et al.* Transplantation of autologously derived mitochondria protects the heart from ischemia–reperfusion injury. *Am J Physiol Heart Circ Physiol* 2013;**304**:H966–H982.

34 J. C. Chang, K. H. Liu, Y. C. Li, *et al.* Functional recovery of human cells harbouring the mitochondrial DNA mutation MERRF A8344G via peptide-mediated mitochondrial delivery. *Neurosignals* 2013;**21**:160–173.

35 S. R. Baglio, D. M. Pegtel, and N. Baldini. Mesenchymal stromal cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012;**3**:359.

36 S. Abounit and C. Zurzolo. Wiring through tunneling nanotubes – from electrical signals to organelle transfer. *J Cell Sci* 2012;**125**:1089–1098.

37 H. R. Chinnery, E. Pearlman, and P. G. McMenamin. Cutting edge: membrane nanotubes *in vivo*: a feature of MHC class $II^+$ cells in the mouse cornea. *J Immunol* 2008;**180**:5779–5783.

38 X. Wang and H. H. Gredes. Long-distance electrical coupling via tunneling nanotubes. *Biochim Biophys Acta* 2012;**1818**:2082–2086.

39 A. R. Williams and J. M. Hare. Mesenchymal stromal cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res* 2011;**109**:923–940.

40 A. Uccelli, V. Pistoia, and L. Moretta. Mesenchymal stromal cells: a new strategy for immunosuppression? *Trends Immunol* 2007;**28**:219–226.

41 B. Parekkadan and J. M. Milwid. Mesenchymal stromal cells as therapeutics. *Annu Rev Biomed Eng* 2010;**12**:87–117.

42 A. Acquistapace, T. Bru, P. F. Lesault, *et al.* Human mesenchymal stromal cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer. *Stem Cells* 2011;**29**:812–824.

43 H. S. Habib, T. F. Halawa, and H. M. Atta. Therapeutic applications of mesenchymal stroma cells in pediatric diseases: current aspects and future perspectives. *Med Sci Monit* 2011;**17**:RA233–RA239.

44 Y. M. Cho, J. H. Kim, M. Kim, *et al.* Mesenchymal stromal cells transfer mitochondria to the cells with virtually no mitochondrial function but not with pathogenic mtDNA mutations. *PLoS ONE* 2012;**7**:e32778.

45 G. Hansmann, A. Fernandez-Gonzalez, M. Aslam, *et al.* Mesenchymal stromal cell-mediated reversal of bronchopulmonary dysplasia and associated pulmonary hypertension. *Pulm Circ* 2012;**2**:170–181.

46 R. L. Toonkel, J. M. Hare, M. A. Matthay, *et al.* Mesenchymal stromal cells and idiopathic pulmonary fibrosis. Potential for clinical testing. *Am J Respir Crit Care Med* 2013;**188**:133–140.

47 Y. G. Zhu, Q. Hao, A. Monsel, *et al.* Adult stem cells for acute lung injury: remaining questions and concerns. *Respirology* 2013;**18**:744–756.

48 K. Liu, K. Ji, L. Guo, *et al.* Mesenchymal stromal cells rescue injured endothelial cells in an *in vitro* ischemia–reperfusion model via tunneling nanotube like structure-mediated mitochondrial transfer. *Microvasc Res* 2014;**92**:10–18.

49 E. Y. Plotnikov, T. G. Khryapenkova, A. K. Vasileva, *et al.* Cell-to-cell cross-talk between mesenchymal stromal cells and cardiomyocytes in co-culture. *J Cell Mol Med* 2008;**12**:1622–1631.

50 M. Koyanagi, R. P. Brandes, J. Haendeler, *et al.* Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? *Circ Res* 2005;**96**:1039–1041.

51 K. C. Vallabhaneni, H. Haller, and I. Dumler. Vascular smooth muscle cells initiate proliferation of mesenchymal stromal cells by mitochondrial transfer via tunneling nanotubes. *Stem Cells Dev* 2012;**21**:3104–3113.

52 H. J. Lee, J. K. Lee, H. Lee, *et al.* The therapeutic potential of human umbilical cord blood-derived mesenchymal stromal cells in Alzheimer's disease. *Neurosci Lett* 2010;**481**:30–35.

53 N. Joyce, G. Annett, L. Wirthlin, *et al.* Mesenchymal stromal cells for the treatment of neurodegenerative disease. *Regen Med* 2010;**5**:933–946.

54 C. Boucherie, S. Schäfer, P. Lavand'homme, *et al.* Chimerization of astroglial population in the lumbar spinal cord after mesenchymal stromal cell transplantation prolongs survival in a rat model of amyotropic lateral sclerosis. *J Neurosci Res* 2009;**87**:2034–2046.

55 L. A. Ortiz, F. Gambelli, C. McBride, *et al.* Mesenchymal stromal cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**:8407–8411.

56 K. Sinclair, S. T. Yerkovich, and D. C. Chambers. Mesenchymal stromal cells and the lung. *Respirology* 2013;**18**:397–411.

57 N. Souidi, M. Stolk, and M. Seifert. Ischemia–reperfusion injury: beneficial effects of mesenchymal stromal cells. *Curr Opin Organ Transplant* 2013;**18**:34–43.

58 X. Li, Y. Zhang, S. C. Yeung, *et al.* Mitochondrial transfer of induced pluripotent stem cell-derived mesenchymal stromal cells to airway epithelial cells attenuates cigarette smoke-induced damage. *Am J Respir Cell Mol Biol* 2014;**51**:455–465.

59 A. Cselenyák, E. Pankotai, E. M. Horváth, *et al.* Mesenchymal stromal cells rescue cardiomyoblasts from cell death in an *in vitro*

ischemia model via direct cell-to-cell connections. *BMC Cell Biol* 2010;**11**:29.

60 J. L. Carvalho, V. B. Braga, M. B. Melo, *et al.* Priming mesenchymal stromal cells boosts stem cell therapy to treat myocardial infarction. *J Cell Mol Med* 2013;**17**:617–625.

61 M. Giuliani, N. Oudrhiri, Z. M. Noman, *et al.* Human mesenchymal stromal cells derived from induced pluripotent stem cells down-regulate NK-cell cytolytic machinery. *Blood* 2011;**118**: 3254–3262.

62 Y. Wang, X. Chen, W. Cao, and Y. Shi. Plasticity of mesenchymal stromal cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* 2014;**15**:1009–1016.

63 F. E. Ezquer, M. E. Ezquer, D. B. Parrau, *et al.* Systemic administration of multipotent mesenchymal stromal cells reverts hyperglycemia and prevents nephropathy in type 1 diabetic mice. *Biol Blood Marrow Transplant* 2008;**14**:631–640.

64 S. Kadam, S. Muthyala, and P. Nair. Human placenta-derived mesenchymal stromal cells and islet-like cell clusters generated from these cells as a novel source for stem cell therapy in diabetes. *Rev Diabet Stud* 2010;**7**:168–182.

65 D. J. Weiss, R. Casaburi, R. Flannery, *et al.* A placebo-controlled, randomized trial of mesenchymal stromal cells in COPD. *Chest* 2013;**143**:1590–1598.

66 M. A. Antunes, J. G. Laffey, P. Pelosi, and P. R. Rocco. Mesenchymal stromal cell trials for pulmonary diseases. *J Cell Biochem* 2014;**115**:1023–1032.

67 G. M. Forbes, M. J. Sturm, R. W. Leong, *et al.* A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* 2014; **12**:64–71.

68 A. F. Macaskill, J. E. Rinholm, A. E. Twelvetrees, *et al.* Miro 1 is a calcium sensor for glutamate receptor-dependent localization of mitochondria at synapses. *Neuron* 2009;**61**:541–555.

69 E. E. Glater, L. J. Megeath, R. S. Stowers, and T. L. Schwarz. Axonal transport of mitochondria requires milton to recruit kinesin heavy chain and is light chain independent. *J Cell Biol* 2006;**173**:545–557.

70 T. T. Nguyen, S. S. Oh, D. Weaver, *et al.* Loss of Miro 1-directed mitochondrial movement results in a novel murine model for neuron disease. *Proc Natl Acad Sci U S A* 2014;**111**: E3631–E3640.

71 X. Wang, D. Winter, G. Ashrafi, *et al.* PINK1 and Parkin target Miro for phosphorylation and degradation to arrest mitochondrial motility. *Cell* 2011;**147**:893–906.

72 Q. Lian, Y. Zhang, J. Zhang, *et al.* Functional mesenchymal stromal cells derived from human induced pluripotent stem cells attenuate limb ischemia in mice. *Circulation* 2010;**121**:1113–1123.

CHAPTER 11

# The regulation of adipogenesis from adipose-derived stem/stromal cells

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## 11.1 Introduction

Adipose tissue or fat is a loose connective tissue in the body composed mostly of adipocytes (fat cells). There are at least three types of adipose tissue: white adipose tissue stores energy and brown adipose tissue generates body heat. Brown adipocytes are located in dedicated depots and express constitutively high levels of thermogenic genes, whereas inducible "brown-like" adipocytes, also known as beige cells, develop in white fat in response to various activators. The activities of brown and beige fat cells reduce metabolic disease (including obesity) in mice and correlate with leanness in humans. Many genes and pathways that regulate brown and beige adipocyte biology have now been identified.

In addition to adipocytes, adipose tissue contains the stromal vascular fraction of cells, which comprises preadipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cells, such as adipose tissue macrophages. Adipose tissue is derived from preadipocytes. Its main role is to store energy in the form of lipids, although it also cushions and insulates the body. Fat cells have an important physiological role in maintaining triglyceride and free fatty acid levels, as well as determining insulin resistance. Abdominal fat has a different metabolic profile, being more prone to induce insulin resistance. Adipose tissue has been recognized as a major endocrine organ, since it produces hormones and other proteins, including leptin, estrogen, estradiol, resistin, adiponectin, plasminogen activator inhibitor-1, tumor necrosis factor-α (TNF-α), interleukin-6, and monocyte chemoattractant protein 1. Adipose tissues also secrete types of cytokines called adipokines (adipocytokines), which play a role in obesity-associated complications. Perivascular adipose tissue releases adipokines, such as adiponectin, that affect the contractile function of the vessels that they surround. Moreover, adipose tissue can affect other organ systems of the body and may lead to disease. White adipose tissue is emerging as an active participant in regulating physiologic and pathologic processes, including immunity and inflammation. Macrophages are components of adipose tissue and actively participate in its activities. Furthermore, crosstalk between lymphocytes and adipocytes can lead to immune regulation.

Obesity is a condition in which a person has an abnormally high and unhealthy proportion of body fat. It is a metabolic syndrome that has become a major public health concern and which presents a formidable therapeutic challenge as well as being a major socioeconomic problem [1]. To measure obesity a scale known as the body mass index (BMI) is used. BMI is

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calculated by dividing a person's weight in kilograms by their height in meters squared. BMI provides a more accurate measure of obesity or being overweight than weight alone. Results from the 2007–2008 US National Health and Nutrition Examination Survey showed that 68% of adults aged 20 years and older in the USA were overweight or obese.

It is widely accepted that obesity is associated with an increased incidence of type II diabetes mellitus, hyperlipidemia, fatty infiltration of the liver (steatosis), cardiovascular disease, and osteoarthritis as well as a number of malignancies, including cancer of the esophagus, breast (after the menopause), colon and rectum, thyroid, endometrium, kidney, and gall bladder [1].

Within adipose tissue, adipocytes are derived from adipose-derived stem/stromal cells (ASCs). ASCs have a propensity for adipogenic differentiation and play a key role in the generation and metabolism of adipose tissue [2,3]. Therefore, understanding the molecular events involved in adipogenesis may contribute to the development of preventive measures and treatments for obesity-related metabolic diseases.

Since ASCs were successfully isolated from adipose tissue by Zuk *et al.* in 2001 [3] much research has been done on their multipotency and on adipogenic differentiation mechanisms. ASCs are easy to harvest with a minor degree of invasiveness, proliferate readily, and their default differentiation pathway is adipogenesis [4–7]. In addition, ASCs can be easily genetically modified with high transfection efficiency and stable gene expression. Thus, ASCs are useful cells for the study of adipogenic differentiation mechanisms [8,9], and it is pertinent to review current knowledge of the regulatory mechanisms in adipogenesis using ASCs.

## 11.2 Adipose-derived stem/stromal cells

### 11.2.1 Preparation and molecular characterization of adipose-derived stem/stromal cells

Adipose tissue consists of many kinds of cells, including mesenchymal stromal cells, preadipocytes, mature adipocytes, fibroblasts, vascular endothelial cells, macrophages, and lymphocytes [10–12]. The stromal-vascular cell fraction of adipose tissue has become the focus of research because it is a rich source of pluripotent cells: ASCs [3,13]. Other names for these cells include adipose-derived adult stromal cells, adipose-derived adult stem cells, adipose mesenchymal stem cells, adipose stromal cells (also abbreviated ASCs), preadipocytes, and ASCs, thus creating some confusion. To minimize confusion, the term adipose-derived stromal/stem cells (ASCs) was recommended by the International Fat Applied Technology Society (IFATS) to describe the plastic-adherent, *ex vivo* expanded and serially passaged multipotent cell population derived from adipose tissue [14–16]; therefore, ASCs will be used throughout this chapter to identify this population of cells.

Adipose tissue can be obtained by surgical resection, tumescent lipoaspiration, or ultrasound-assisted lipoaspiration. The word "tumescent" means swollen and firm. This technique involves subcutaneous infiltration of large volumes of crystalloid fluid that contains low concentrations of epinephrine (adrenaline) and the local anesthetic lignocaine followed by suction-assisted aspiration of fat using small aspiration cannulae called microcannulae. The term tumescent liposuction specifically excludes the use of any additional anesthesia, either intravenous or gaseous, and by definition is a method for performing liposuction surgery with the patient using local anesthesia only. The principal protocols can be obtained from the literature [17–19], and the ASC harvest procedures are shown in Figure 11.1.

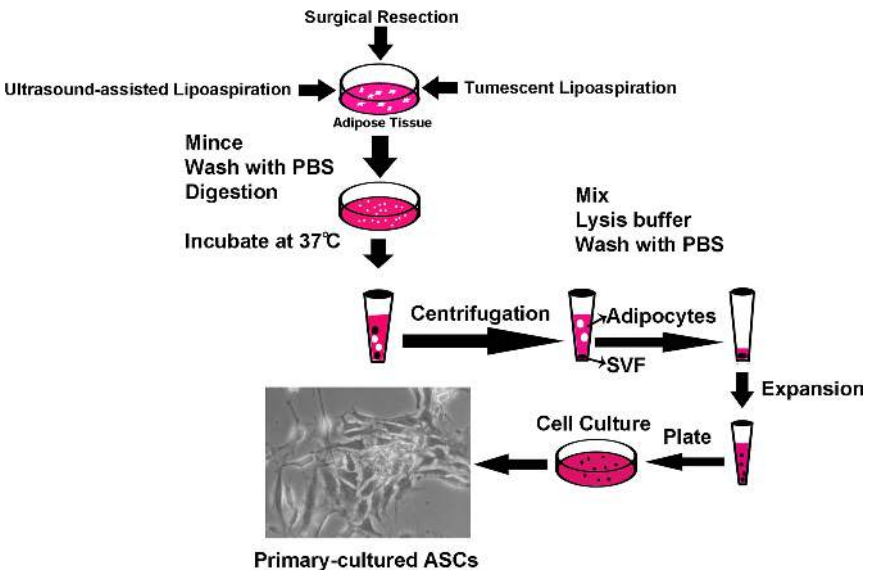



**Figure 11.1** Schematic diagram of the preparation and isolation of ASCs. Adipose tissue can be obtained by surgical resection, tumescent lipoaspiration, or ultrasound-assisted lipoaspiration. The adipose tissue is minced, incubated at 37 °C for 40 min with 0.075% type I collagenase and then centrifuged at 1000 rpm for 5 min. After washing with phosphate-buffered saline (PBS) and expanded in α-minimum essential medium with 10% fetal bovine serum, the cells are cultured in a 5% $CO_2$ humidified atmosphere. Finally, cells are identified by their multilineage differentiation potential, cell surface antigen profile, plastic adherence ability, and colony formation ability. SVF: stromal vascular fraction.

It should be noted that there are discrepancies in the results obtained from different laboratories. There are a number of potential reasons for this. First, ASCs are isolated from donors who differ in ethnicity, medical history, age, gender, and BMI. One study reported that BMI negatively correlated to the number of stromal cells per unit volume and their differentiation capacity [17]. Second, it has been reported that operative site and surgical procedure are relevant to the proliferation and differentiation capacity of ASCs. Third, the quality of ASC isolation and the culture procedure for isolating ASCs differ between laboratories [18,19]. These variables may affect the composition of the initial cell population isolated, but it is difficult to standardize these variables. In addition, other variables such as initial cell plating density and confluence, coating of culture dishes, cell culture media, cell culture environment, and cell subculture procedures may influence the ASC population obtained.

Studies have shown that ASCs in passage 2 or 3 have a distinct cell surface phenotype and express CD10, CD13, CD29, CD44, CD49e, CD73, CD90, CD105, and CD166 and do not express CD11b, CD14, CD31, and HLA-DR [20,21]. However, some surface markers, including CD29, CD90, and CD166, increase during culture [14], while other markers, such as CD34, decrease. It has been reported that CD34 is expressed in the initial cultures of ASCs, but decreases dramatically during culture [22]. Therefore, it is necessary to characterize the expression profile of ASCs at different times in culture and during different passages.

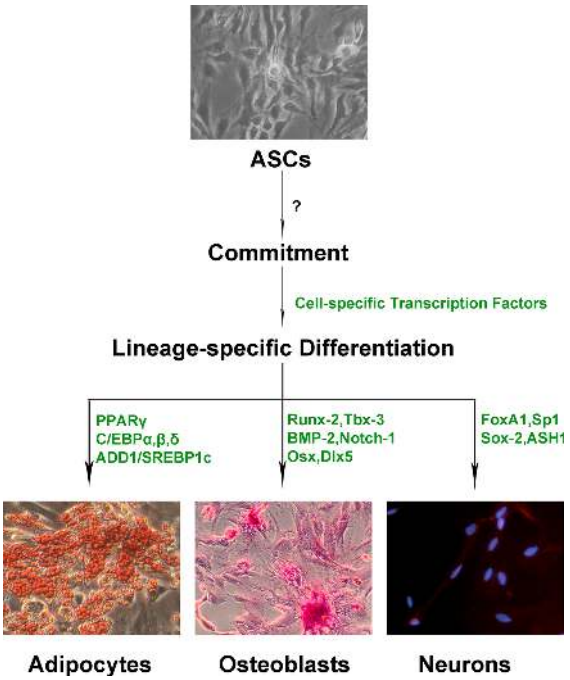



**Figure 11.2** The multilineage differentiation model of ASCs. The mechanisms by which ASCs commit to the adipogenic lineage are poorly understood, although lineage-specific terminal differentiation is regulated by cell-specific transcription factors. The adipogenic, osteogenic, and neurogenic differentiations of ASCs are shown. (See color plate section.)

### 11.2.2 Differentiation capacity of adipose-derived stem/stromal cells

ASCs have the capacity to differentiate into cells of the mesodermal lineage, including adipocytes, fibroblasts, myocytes, osteocytes, and chondrocytes [23]. Overexpression of peroxisome proliferator-activated receptor-γ (PPAR-γ) in ASCs results in adipogenic differentiation. Surprisingly, there appears to be evidence that ASCs also have the ability to differentiate into non-mesodermal cells, including hepatocytes, neurons, pancreatic cells, endothelial cells, and cardiomyocytes [24–27]. However, before initiating lineage-specific differentiation, ASCs have to be "allocated" or "committed" to a certain lineage. Although several studies have investigated the commitment process and the mechanisms by which pluripotent stem cells commit to a certain lineage, little information is available as yet [28,29]. The multilineage differentiation model of ASCs is shown in Figure 11.2.

## 11.3 Process of adipogenic differentiation from adipose-derived stem/stromal cells

### 11.3.1 Adipocyte development program

#### 11.3.1.1 Commitment

The initial stage of commitment to adipogenic differentiation is ASCs differentiating into adipocyte precursor cells and the subsequent differentiation of adipocyte precursor cells into preadipocytes. The mechanisms of this process are poorly understood [15,29]. To date, no specific adipogenic commitment factor has been found. There is some evidence that adipogenic orientation may be associated with cytokines secreted by the mature adipocytes, but no specific factors have been identified [30,31]. Tang *et al.* [32] reported that bone morphogenetic protein 4 (BMP4) induced the commitment of the pluripotent cells C3H10T1/2 to preadipocytes, suggesting that BMP4 may be involved in the commitment process of adipogenic differentiation. (The C3H10T1 cell line was established in 1973 from 14- to 17-day-old C3 H mouse embryos. These cells display fibroblastic morphology in cell culture and are functionally similar to mesenchymal stem cells.)

McBeath *et al.* [33] found that ASCs cultured in high density with cell-to-cell contact and no room to "stretch" showed a tendency to adipogenic differentiation, whereas cells plated at low density or on a large island of fibronectin, which allowed them to spread, were more likely to become osteoblasts. Those studies suggest that cell density and cell morphology may be related to lineage commitment.

#### 11.3.1.2 Growth arrest and mitotic clonal expansion

When ASCs differentiate into preadipocytes, cell confluence and contact inhibition occur, making the cell cycle stop in the $G_0/G_1$

phase [34]. Cells subsequently reenter the cell cycle by induction of lipogenic hormones and mitogenic compounds and undergo several rounds of DNA replication and mitosis, resulting in an increase in cell number. This process is called mitotic clonal expansion. In this process, DNA replication and changes in chromosome conformation first induce the expression of CCAAT (cytosine–cytosine–adenosine–adenosine–thymidine) enhancer binding proteins (C/EBP) β and δ and subsequently the core and crucial transcription factors C/EBPα and PPAR-γ are expressed. Once C/EBPα and PPAR-γ are expressed, various other adipocyte-related genes, such as adipocyte protein 2 (AP2) and glucose transporter (GLUT) 4, begin to be expressed [34,35]. With the increase of C/EBPα and PPAR-γ, cyclin dependent kinase inhibitor p21 is upregulated, cells gradually stop clonal expansion, exit from the cell cycle, and reach a terminal differentiation stage [36–38]. A number of studies have demonstrated that inhibition of clonal expansion or blockade of the cell cycle impair adipogenesis [39–42], while other studies have reported that mitotic clonal expansion is not required in adipocyte development [43,44].

#### 11.3.1.3 Terminal differentiation

Following exit from clonal expansion, cells enter an irreversible terminal differentiation stage. Lipoprotein lipase (LPL) is considered to be the marker of the terminal stage of adipogenic differentiation [34], although some studies indicate that LPL is not a fat-specific marker because of its expression in myocardial cells and macrophages [45,46]. At this stage, C/EBPα and PPAR-γ act synergistically to activate the adipocyte-specific genes that produce the adipocyte phenotype [47]. Subsequently, lipid droplets begin to form and gradually accumulate, and cell shape becomes rounded rather than its previous fibroblast-like appearance. With the enlargement of fat droplets, the cells are gradually filled with a single and large lipid droplet, and the cytoplasm and nucleus are extruded to one side. At the same time, many adipocyte-specific genes are expressed, which enhance the sensitivity of the cells to insulin. After this the adipocytes begin to secrete a variety of adipokines or cytokines, culminating in mature adipocyte differentiation [2,34,42]. This process of adipogenic differentiation from ASCs is illustrated in Figure 11.3.

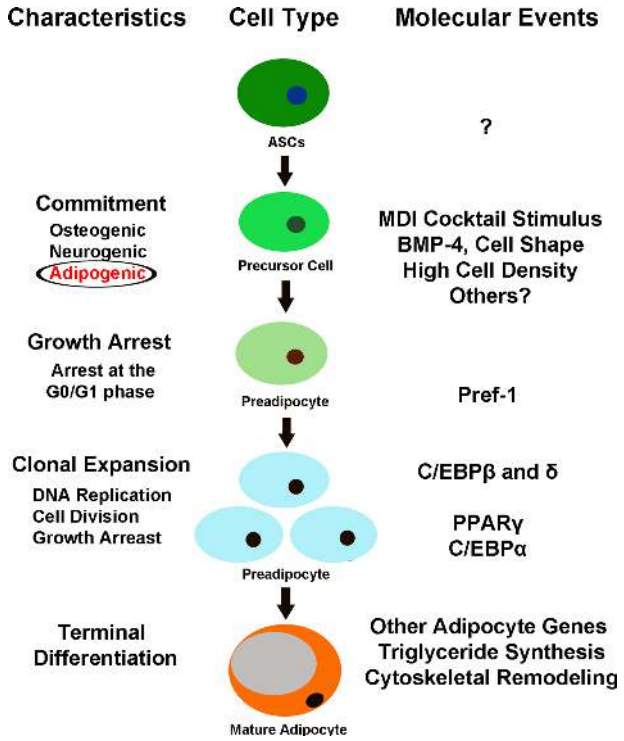



**Figure 11.3** The adipogenic differentiation process of ASCs. ASCs commit to precursor cells and subsequently differentiate to preadipocytes induced by 3-isobutyl-1-methylxanthine, dexamethasone, and insulin (MDI) cocktail, BMP-4, high cell density, and cell shape. Preadipocytes reach confluence and arrest at $G_0/G_1$ phase. These growth-arrested cells subsequently reenter the cell cycle and undergo several rounds of cell division, referred to as mitotic clonal expansion, during which cells gradually express C/EBPs and PPARs. After the clonal expansion process, cells exit the cell cycle and reenter a unique growth-arrested stage. Subsequently, they commit to their terminal differentiation stage, during which they express many other adipocyte-specific genes, increase glucose uptake, and enable fat accumulation.

### 11.3.2 Signaling pathways associated with adipogenic differentiation

#### 11.3.2.1 The PI3K/Akt signaling pathway

Insulin-like growth factor-1 (IGF-1) was the first reported cytokine able to promote adipocyte development. It activates phosphatidylinositol (−3) kinase (PI3K) through binding to the IGF-1 receptor and further activates the serine/threonine protein kinase Akt, leading to activation of the PI3 K/Akt pathway. Once the PI3 K/Akt pathway is activated, GLUT1 and −4 translocate to the cell membrane and glucose is absorbed into cells to synthesize triglyceride, which promotes the maturation of adipocytes [48,49]. A previous study showed that activating Akt can promote the differentiation of 3T3-L1 cells into mature adipocytes, while RNA interference (RNAi)-mediated inhibition of Akt1 expression can block the adipogenic process [50]. Embryonic stem cells from Akt1 knockout mice are unable to express PPAR-γ and thus cannot differentiate into adipocytes [51,52]. In contrast, exogenous upregulation of PPAR-γ in Akt1 knockout stem cells enables the adipogenesis process, suggesting that PPAR-γ may be the direct downstream gene of Akt1 [52,53].

#### 11.3.2.2 The cAMP/PKA/CREB signaling pathway

The key activation event of the cyclic AMP response element-binding protein (CREB) is phosphorylation on the Ser 133 site. G protein–cyclic adenosine monophosphate–protein kinase A (G protein–cAMP–PKA) is the classical pathway that acts on CREB. Activated PKA enters the nucleus and

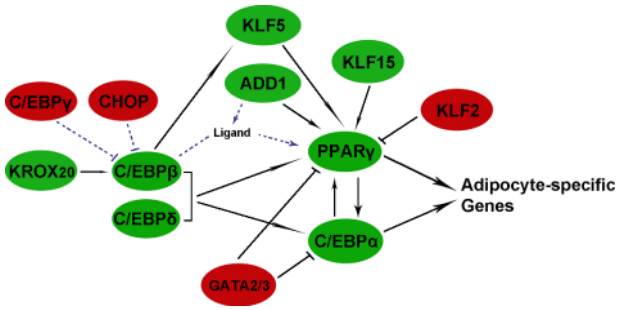



**Figure 11.4** The transcriptional cascade that regulates adipogenesis is shown. PPAR-γ and C/EBPα lie at the core of the transcriptional cascade that regulates adipogenesis. The transcription factors in light gray ovals are anti-adipogenesis and the transcription factors in dark gray ovals are pro-adipogenesis. Solid lines indicate effects on gene expression, whereas dashed lines represent effects on protein activity.

phosphorylates CREB. Homodimers of phosphorylated CREB play a role in regulating the transcription of target genes by binding to their cAMP response elements with the associated factor phosphorylated CREB binding protein [54]. Expression of constitutively active CREB strongly activates C/EBPβ promoter–reporter genes, which induce expression of endogenous C/EBPβ and results in adipogenesis. Conversely, expression of a dominant-negative CREB impairs promoter–reporter activity, expression of C/EBPβ, and adipogenesis [54,55] (Figure 11.4).

#### 11.3.2.3 The MAPK signaling pathway

Mitogen-activated protein kinase (MAPK) is another member of the serine/threonine protein kinase family. The MAPK signaling pathway is involved in cell proliferation, cell differentiation, and cell apoptosis. The MAPK signaling pathway is comprised of three sequentially activated kinases and regulated by phosphorylation. MAPK kinase kinases (MKKKs) are the first component of this phosphorelay system. MAPK kinases (MKKs) serve as the phosphorylation substrates of MKKKs. MAPKs are the downstream component activated by MKKs. The MAPK signaling pathway in mammals has three well-characterized subfamilies: the extracellular signal-regulated kinases (ERKs), the c-Jun NH2-terminal kinases, and the p38 enzymes [56,57]. The role of the MAPK signaling pathway in the process of adipocyte differentiation is complex and depends on the phase of cell differentiation. When cell differentiation is in the phase of clonal expansion, the MAPK signaling pathway is required for mitosis, whereas when cells enter their terminal differentiation stage the MAPK signaling pathway is inactivated so that MAPKs can phosphorylate PPAR-γ, forming inactivated p-PPAR-γ, an inhibitor of adipogenesis [40,58,59]. Additionally, the role of MAPKs may be different in different species and in different cellular models [60].

#### 11.3.2.4 The Wnt/β-catenin signaling pathway

The Wnt/β-catenin pathway is also involved in the regulation of adipogenic differentiation. β-catenin plays a key role as a second messenger in this process [61,62]. When the Wnt/β-catenin pathway is activated, Wnt proteins such as Wnt10B bind to its specific receptor Frizzled and its auxiliary receptor low density lipoprotein receptor-related protein, inactivating Dishevelled protein, which has an inhibitory role on glycogen synthase kinase-3β, cytoskeletal protein (Axin), and colorectal adenoma-like protein (APC). Thus, β-catenin is not phosphorylated, permitting the binding of activated β-catenin and lymphoid enhancer-binding factor (LEF) and Transcription Factor (TCF) family members, which in turn triggers the expression of downstream genes. These activated downstream genes inhibit the expression of PPAR-γ and C/EBPα, thereby inhibiting adipogenesis [63,64].

#### 11.3.2.5 The TGF-β signaling pathway

The transforming growth factor-β (TGF-β) pathway initiates signaling by binding to transforming growth factor beta type I and type II receptor kinases (TβRI and TβRII). Certain TGF-β superfamily members, including TGF-β, bone morphogenetic proteins (BMPs), and activins, may initiate signaling by binding to TβRII, which leads to the recruitment of an appropriate TβRI. Activated TβRs induce the phosphorylation of receptor-associated SMADs such as SMAD2 and SMAD3. (The SMAD proteins are homologs of both the *Drosophila* protein, mothers against decapentaplegic (MAD), and the *Caenorhabditis elegans* protein SMA – from gene SMA for small body size. The name is a portmanteau of the two.) Activated SMAD2 and SMAD3 bind to SMAD4. The compound thus formed translocates to the nucleus and binds to DNA binding assistant factors to regulate transcription [65]. TβRs can also activate other signaling pathways, such as the MAPK signaling pathway [66,67]. Previous studies have shown that TGF-β inhibited adipogenic differentiation of primary cultured preadipocyte *in vitro* and *in vivo* [68–70]. Overexpression of TβRI in adipose tissue severely blocks both white and brown adipose tissue formation [71].

## 11.4 Regulation of adipogenic differentiation from adipose-derived stem/stromal cells

### 11.4.1 Transcriptional regulation

#### 11.4.1.1 The PPAR family

The transcriptional activation role of PPARs, a subgroup of nuclear hormone receptors, on adipogenic differentiation has been widely confirmed. It has been shown that PPARs can induce adipogenic differentiation, not only of ASCs and preadipocytes [72–75] but also myoblasts [76]. PPARs have three subtypes: PPAR-α, PPAR-δ, and PPAR-γ. They are

directly regulated by steroids, thyroid hormones, vitamins, lipid metabolites, and xenobiotics [77]. (A xenobiotic is a foreign chemical substance found within an organism that is not normally naturally produced by, or expected to be present within, that organism. It can also cover substances that are present in much higher concentrations than are usual. Specifically, drugs such as antibiotics are xenobiotics in humans because the human body does not produce them itself, nor are they part of a normal food.) PPAR-α and PPAR-δ are not only expressed in adipose tissue, but also in other tissues and cell lines [78,79]. Compared with the high level of PPAR-α in brown adipose tissue, the level in white adipose tissue and adipocyte cell lines is extremely low [80,81], suggesting that the role of PPAR-α in white adipose tissue or in adipocytes from white adipose tissue is minor. PPAR-γ is expressed almost exclusively in adipose tissue and adipocyte cell lines and is hardly expressed at all in other tissues and cell lines [42,47,82]. The role of PPAR-α and PPAR-γ as the critical regulators of adipogenesis has been supported by overwhelming evidence from both *in vivo* and *in vitro* studies [2,82–84]. PPAR-α and PPAR-γ are induced in the early phase of the adipogenic differentiation process. Once expressed they ensure that terminal differentiation is maintained and can activate adipocyte-specific genes, producing the adipocyte phenotype [47,82].

#### 11.4.1.2 The C/EBP family

The C/EBP family has three isoforms: C/EBPα, C/EBPβ, and C/EBPδ. All of them possess C-terminal basic region/leucine zipper domains that confer DNA-binding ability and the ability to dimerize. Many studies have confirmed that the C/EBP family plays an important role in adipogenesis. The expression of C/EBPα occurs prior to the expression of most adipocyte-specific genes. Gain-of-function studies demonstrated that expression of C/EBPα was sufficient to induce adipogenic differentiation of preadipocytes without the addition of external hormonal inducers. Disruption of C/EBPα resulted in failure to develop white adipose tissue in mice [34,85]. Additionally, C/EBPα can maintain the terminally differentiated state through autoactivation of its own gene in the differentiation process [86,87]. An increasing body of evidence indicates that C/EBPβ and C/EBPδ function in the early stage of the adipogenic differentiation process and are expressed prior to C/EBPα as transcriptional activators in the sequence of events leading to adipogenic differentiation. Adipogenic induction on growth-arrested preadipocytes provokes an immediate and transient burst of expression of C/EBPβ and C/EBPδ [88]. Subsequently, the preadipocytes reenter the cell cycle, undergoing several rounds of mitotic clonal expansion and expressing C/EBPα, which arrests growth, and a return to terminal differentiation ensues [34,47]. Generally, C/EBPβ is regarded as the transcriptional activator of the C/EBPα gene and C/EBPδ may act synergistically with C/EBPα in the induction of PPAR-γ [89]. The transcriptional cascade that regulates adipogenesis has been reviewed by Rosen and MacDougald [35], and a schematic diagram is shown in Figure 11.4.

#### 11.4.1.3 ADD1/SREBP-1c

Adipocyte determination and differentiation-dependent factor 1 (ADD1), also called sterol regulatory element binding protein 1 (SREBP-1), is a transcription factor that regulates the synthesis of unsaturated fatty acids, glucose metabolism, and the synthesis of cholesterol [90]. SREBPs have three isoforms: SREBP-1a, SREBP-1c, and SREBP-2. SREBP-1c is highly expressed in white adipose tissue, brown adipose tissue, and liver cells, with the capacity of promoting fat synthesis in the liver and adipogenic differentiation of 3T3-L1 preadipocytes. There are two main ways by which SREBP-1c promotes the synthesis of triglyceride in the liver and adipocytes: first, by upregulating the expression of fatty acid synthetase, acetyl coenzyme A decarboxylase, stearoyl coenzyme A, and phosphoenolpyruvate carboxykinase to promote triglyceride synthesis [91]; second, by transcription of activating PPAR-γ or induction of the expression of the ligand of PPAR-γ to promote adipogenic differentiation [92]. Although details of the regulatory mechanism of ADD1/SREBP-1c in adipocyte differentiation have not been fully elucidated, many studies suggest that ADD1/SREBP-1c plays an important role in the development of adipocytes. In addition, the role of ADD1/SREBP-1c in the differentiation process of adipocytes from brown adipose tissue remains unclear.

### 11.4.2 Epigenetic regulation

#### 11.4.2.1 DNA methylation

DNA methylation was the first recognized epigenetic mechanism and the one that has been the most extensively studied. DNA methylation in mammals is the process by which methyl from *S*-adenosylmethionine is transferred to a specific nucleotide, usually at position 5 of cytosine, and catalyzed by DNA methyltransferase. Generally, DNA methylation occurs on CpG islands, but it also occasionally occurs on in a CpHpG context where H = A (adenosine), T (thymine), or C (cytosine) [93]. DNA methylation does not alter DNA sequence, but it is an important epigenetic modification in the maintenance of normal cell function, genetic imprinting, embryonic development and human oncogenesis [94]. The main role of DNA methylation is the regulation of gene expression, and it is one of the most important epigenetic mechanisms of this. It has been found that DNA methylation plays an important role in development of adipose tissue and occurrence of obesity [95].

##### *11.4.2.1.1 DNA methylation at the genomic level*

Zhu and colleagues analyzed human genomic DNA methylation in preadipocytes and mature adipocytes and found that hypomethylation occurred on 2701 genes and that hypermethylation occurred on 1070 genes [96]. This suggests that genomic DNA

methylation is involved in adipogenic differentiation. Further analysis found that the genes with methylation were mainly those of the signaling pathways of inflammatory cytokines, the Wnt signaling pathway, the MAPK signaling pathway, and insulin-induced signaling pathways. Signaling pathway network analysis showed that TNF-α and interleukin-8 played a key role in this network [97].

#### *11.4.2.1.2* C/EBP *methylation*

Li *et al.* found that hypomethylation occurred on the *C/EBPα* gene in preadipocytes, while hypermethylation occurred on the *C/EBPα* gene in mature adipocytes, suggesting that DNA methylation regulates adipocyte development [97]. Another study found that the levels of DNA methylation on the promoter region of *C/EBPα* were significantly elevated after contact inhibition of 3T3-L1 cells for 48 h. However, when cells were induced by the cocktail of isobutylmethylxanthine, dexamethasone, and insulin for 24 h the level of DNA methylation on the promoter region of *C/EBPα* was downregulated. If the cells were treated with insulin only, the methylation of *C/EBPα* was maintained at a high level. Meanwhile, the expression of C/EBPα was upregulated when induced with isobutylmethylxanthine, dexamethasone, and insulin, whereas there was almost no change when induced with insulin only [96]. These results indicate that the commitment of preadipocytes may be associated with DNA methylation and that this epigenetic modification for cell commitment promotes genetic stability.

#### *11.4.2.1.3* PPAR-γ *methylation*

In contrast to the effect of DNA methylation of *C/EBPα*, Noer *et al.* found that hypomethylation occurred on the promotor region of *PPAR-γ* in mesenchymal stem cells, whereas hypermethylation occurred on the promotor region of *PPAR-γ* in preadipocytes, and demethylation gradually occurred as adipogenic differentiation got under way [98]. Additionally, the mRNA level of PPAR-γ became elevated, which suggested that the demethylation of *PPAR-γ* can promote preadipocytes to differentiate into mature adipocytes [99]. The DNA methylation-inhibitor 5-azacytidine was capable of downregulating the level of methylation on *PPAR-γ* and increased the expression of *PPAR-γ* [100]. In addition, chromatin immunoprecipitation experiments showed that methyl CpG binding protein 2 had bound to the promoter region of *PPAR-γ* early in the adipogenic commitment process [101]. Interestingly, this study showed that there were some CpG sites 500 base pairs upstream of the transcription start site of human *PPAR-γ*2, but these were absent at the same sites for the mouse *PPAR-γ*2 gene, suggesting that DNA methylation of a given gene may differ in different species [99].

#### *11.4.2.1.4 Leptin methylation*

Leptin is a hormone secreted mainly by adiopcytes. It can inhibit appetite and regulate energy metabolism. One study found that *leptin* expression was regulated by DNA methylation on its promoter region. In mesenchymal stem cells the promoter region of *leptin* was in a hypomethylation state, but in preadipocytes it was in hypermethylation state without *leptin* expression in the cells [98]. Okada *et al.* reported that methylation inhibitor treatment of 3T3-L1 preadipocytes did increase expression of *leptin* mRNA [102]. In other studies of the process of preadipocyte differentiation to mature adipocytes, demethylation of the *leptin* promoter site gradually occurred and the mRNA level of *leptin* increased [103,104]. Milagro *et al.* [105] also found that in adipose tissue of high fat diet-induced obese rats, the methylation level in the promoter region of *leptin* slightly increased. These results suggest that methylation of *leptin* plays a regulatory role in adipogenesis.

#### *11.4.2.1.5 Methylation of* ARHGEF*19*

Ras homolog gene family member A guanine nucleotide exchange factor 19 (ARHGEF19) is a member of the RhoGEFs family. RhoGEFs play an important role in many physiological and pathological processes. RhoGEFs promote conversion of GDP to GTP and produce the active form of GTP enzyme. ARHGEF19 is mainly expressed in the intestine, liver, heart, and kidney [106]. ARHGEF19 can activate RhoA, which is capable of inhibiting adipogenesis through the RhoA-Rho kinase (ROCK) signaling pathway by regulating cytoskeletal tension [33]. Wang *et al.* found that the level of methylation on the promotor region of *ARHGEF19* in adipogenically differentiated 3T3-L1 cells was downregulated compared with that in undifferentiated cells [106]. Additionally, the mRNA level of *ARHGEF19* also decreased markedly, suggesting that methylation of *ARHGEF19* upregulates the transcription of the *ARHGEF19* gene. This seems contrary to the consensus that DNA methylation inhibits gene expression, but other studies have shown that methylation on the 5′ end or 3′ end of the gene can activate gene expression [107].

#### *11.4.2.1.6 Methylation of other adipocyte-specific genes*

GLUT4 can translocate to the cell membrane to participate in the transport of glucose as a result of insulin stimulation. In preadipocytes the promoter region of *GLUT4* is methylated, but demethylation occurs when cells start to differentiate adipogenically. In addition, methylation on specific sites of the promoter region inhibits the binding of nuclear factor and promoter, leading to downregulation of *GLUT4* expression [108]. Additionally, fatty acid binding protein 4 and LPL are highly expressed in adipose tissue. It is hypomethylated on the promotor region of the two genes and is hypomethylated in freshly isolated mesenchymal stem cells, cultured mesenchymal stem/stromal cells, and in differentiated mesenchymal stem/stromal cells [98].

### 11.4.2.2 Histone modification

Histone modifications include acetylation and deacetylation, phosphorylation and dephosphorylation, and methylation




**Figure 11.5** Histone modifications. Acetylation (ac) generally correlates with gene activation. Histone lysine (K) methylations (me) that correlate with gene activation are shown in light script (as opposed to bold script).

and demethylation. These modifications not only lead to conformational change of chromatin by affecting the affinity between histones and the DNA chain, but also affect the binding affinity between transcription factors and the response element in DNA sequences, thus indirectly influencing gene expression and resulting in phenotypic change [109,110]. The commonest histone modification pattern is shown as Figure 11.5.

Kim *et al.* reported that histone deacetylase (HDAC) led to a dramatic attenuation of adipogenic differentiation from preadipocytes [111]. In contrast, sodium butyrate treatment increased adipogenic differentiation, while apicidin treatment decreased the expression of adipogenic marker genes, indicating that the adipogenic differentiation process can be differentially regulated depending on the type of HDAC inhibitor used. Additionally, this differential effect seemed not to be due to disruption of the insulin-signaling pathway. Chatterjee *et al.* found that HDAC9 markedly downregulated adipogenic differentiation [112]. ASCs from HDAC9 gene-knockout mice exhibited accelerated adipogenic differentiation, whereas HDAC9 overexpression in 3T3-L1 preadipocytes suppressed adipogenic differentiation, demonstrating its direct role as a negative regulator of adipogenesis. Yoo *et al.* presented evidence that HDACs are downregulated during adipocyte differentiation [113]. Histone hyperacetylation was selectively induced at the promoter regions of adipocyte-specific genes during adipogenic differentiation, producing a dramatic decrease of HDAC1, −2, and −5 and a reduction of HDAC enzyme activity. Similarly, HDAC1-knockdown promoted adipogenesis, whereas HDAC1 overexpression attenuated adipogenic differentiation in 3T3-L1 cells.

PPAR-γ has two main isoforms: PPAR-γ1 and PPAR-γ2. Although they are transcribed by two different promoters, they are expressed at comparable levels in adipogenesis [114]. Mono-, di-, and trimethylations on histone H3K4 (H3K4me1, H3K4me2, and H3K4me3 respectively) generally correlated with gene activation. Studies showed that the action of H3K4me3 on promotors of PPAR-γ1 and PPAR-γ2 increased the expression levels of both PPAR-γ1 and PPAR-γ2 [115]. In addition, Wang *et al.* reported that decreases of H3K27me3 on promotors of Wnt1, Wnt6, Wnt10A and Wnt10B, which are negative regulators of adipogenesis, increased their expression levels and led to inhibition of adipogenesis [116]. Taken together, these results suggest that histone modification is crucial for the execution of adipogenesis.

## 11.4.3 Post-transcriptional regulation

### 11.4.3.1 Regulation of adipogenesis by microRNAs

#### *11.4.3.1.1 MicroRNAs*

MicroRNAs (miRNAs or miRs) are a class of endogenous noncoding single-stranded RNA of 21–25 nucleotides in length [117]. Usually, miRNAs regulate gene expression by binding to complementary target sites in the 3′ untranslated region (3′ UTR) of protein-coding genes [118], although there are a few reports that some miRNAs can activate translation as well [119,120]. miRNAs have several remarkable characteristics: first, they do not have the open reading frame or other characteristics of protein-coding genes [121]; second, most miRNAs are derived from intergenic sequences, although a few are derived from intronic sequences [122–124]; third, the sequences of mature miRNA are highly conserved among different species [125]; and fourth, miRNAs are expressed with high temporal, spatial, and tissue specificity [121].

#### *11.4.3.1.2 MicroRNAs regulate adipogenesis by targeting the PI3K/Akt signaling pathway*

He *et al.* reported that overexpression of miR-29 in diabetic rats sharply repressed insulin-stimulated glucose uptake by downregulating Akt activation, whereas inhibition of miR-29 potentiated Akt phosphorylation, thus augmenting insulin-dependent glucose uptake [126]. The results suggested that miR-29 is capable of decreasing glucose uptake and inhibiting triglyceride formation by impairing the activation of Akt. A study by Ouaamari *et al.* showed that miR-375 can repress the glucose-mediated stimulatory effect on insulin gene expression by targeting phosphoinositide-dependent protein kinase-1 (PDK1), which activates Akt in the presence of phosphatidylinositide 3-kinase (PI3K) [127]. Another study determined that the levels of miR-320 increased approximately 50-fold in insulin-resistant adipocytes. When these adipocytes were transfected with specific antisense (AS) oligonucleotides against miRNA-320, the PI3K/Akt pathway was activated by increasing the expression of PI3K, the phosphorylation of Akt, and the protein expression of GLUT-4, as well as insulin-stimulated glucose uptake. Further study showed that PI3K may be the target of miR-320 [128]. Yi *et al.* reported that miR-143 promoted adipogenesis by targeting pleiotrophin (PTN), which plays a negative role on the PI3K/Akt1 signaling pathway [129]. Jordan found that the expression level of miR-143 was higher in the liver of obese mice [130]. Further study showed that overexpression of miR-143 impairs glucose homeostasis and insulin-stimulated Akt activation via the downregulation of oxysterol-binding-protein-related protein 8. Conversely, a reduction in miR-143 can reduce obesity-associated insulin resistance. Taken together, these studies provide direct evidence that miRNA is capable of regulating adipogenesis by acting on the PI3 K/Akt signaling pathway. The miRNA regulatory PI3 K/Akt signaling pathway of adipogenic differentiation is illustrated in Figure 11.6.

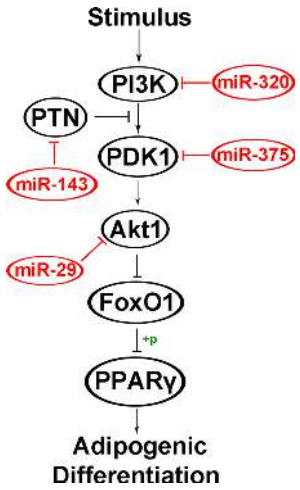



**Figure 11.6** miRNAs regulate adipogenesis by targeting the PI3 K/Akt signaling pathway. miR-320, miR-375, and miR-29 inhibit adipogenic differentiation by targeting PI3 K, PDK1, and Akt1 respectively. miR-143 promotes adipogenesis by targeting PTN, which exerts a negative role during adipogenesis via the PTN/PI3 K/AKT signaling pathway.

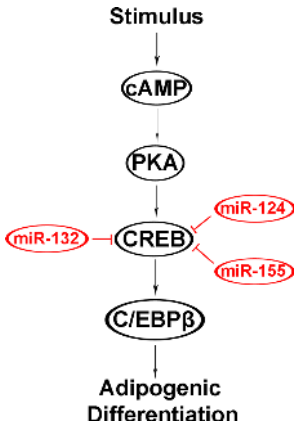



**Figure 11.7** miRNAs regulate adipogenesis by targeting the cAMP/PKA/CREB signaling pathway. cAMP induced by insulin regulates target gene transcription primarily by catalyzing the phosphorylation of CREB through PKA. In adipogenic differentiation, CREB activates C/EBPβ, which triggers adipogenic differentiation. miR-124, miR-132, and miR-155 repress adipogenic differentiation by targeting CREB.

#### *11.4.3.1.3 MicroRNAs regulate adipogenesis by targeting the cAMP/PKA/CREB signaling pathway*

Rajasethupathy *et al.* [131] and Millan [132] found that the brain-specific miRNA miR-124 was able to de-repress the expression of CREB, and a conserved miR-124 target site was found in the 3-UTR of CREB. Downregulation of miR-124 promoted adipogenesis. Liu and colleagues found that when 3T3-L1 preadipocytes were treated with TNF-α, an inhibitor of adipogenesis, miR-155 was up-regulated, while the expression of CREB was reduced [133]. Bioinformatic analysis showed a region in miR-155 complementary to the sequences in 3′ UTR of CREB, suggesting that miR-155 can directly target CREB. Another miRNA that is highly expressed in brain tissue and neuronal cell, miR-132, also plays a role in glucose homeostasis in adipocyte differentiation by regulating the expression of CREB [134]. Therefore, we conclude that miRNAs can regulate adipogenesis by targeting the cAMP/PKA/CREB signaling pathway (Figure 11.7).

#### *11.4.3.1.4 MicroRNAs regulate adipogenesis by targeting the MAPK signaling pathway*

Esau *et al.* first reported that the expression level of miR-143 varied in the adipocyte development process: treatment with AS miR-143 oligonucleotides effectively impaired adipogenic differentiation [135]. The protein level of extracellular signal-regulated kinase 5 (ERK5) that was predicted to be the target gene of miR-143 by bioinformatics analysis increased when the expression of miR-143 was inhibited, suggesting that miR-143 serves as a regulator in adipogenesis and may play a role through acting on ERK5. Similarly, Takanabe *et al.* found that the expression of miR-143 in the mesenteric adipose tissue of mice fed a high fat diet was upregulated, while the protein levels of ERK5 were not significantly downregulated [136]. Oskowitz *et al.* also observed that miR-143 was upregulated during the adipogenic differentiation of human multipotent bone marrow stromal cells and that overexpression of miR-143 promoted adipogenic differentiation [137]. However, the role of ERK5 remains unclear. Chen *et al.* first reported that the regulatory role of miR-143 may depend on the adipogenic differentiation stage [138]. When miR-143 was overexpressed during the clonal expansion stage, the adipogenic differentiation of adipose-derived stromal cells was inhibited, whereas overexpression of miR-143 during the growth arrest stage or terminal differentiation stage promoted differentiation. This study provided evidence that miR-143 plays the role of regulating adipocyte differentiation in part by promoting the transition from the clonal expansion stage to the terminal differentiation stage and validated that miR-143 regulating adipogenesis may act through direct repression of MAP2K5, a key member of the MAPKK family in the MAPK signaling pathway. In addition, Ling *et al.* reported that in 3T3-L1 preadipocytes the expression level of miR-375 was increased by adipogenic induction and that overexpression of miR-375 promoted 3T3-L1 adipogenic differentiation [139]. Further studies found that miR-375 was capable of suppressing phosphorylation levels of ERK1/2, which play an important role in the ERK1/2 signaling pathway. Other miRNAs, such as the *Drosophila* microRNA miR-14, can suppress fat metabolism by targeting the p38 MAPK signaling pathway [140]. In conclusion, miRNAs targeting the MAPK signaling pathway serve as both promoters and inhibitors for adipogenesis depending on which differentiation stage they act on.

High-mobility group AT-hook 2 (HMGA2) is an important architectural transcription factor that is involved in cell

mitosis. The protein belongs to the non-histone chromosomal high-mobility group (HMG) protein family. HMG proteins function as architectural factors and are essential components of the enhanceosome. HMGA2 contains three basic DNA-binding domains (AT-hooks) that cause the protein to bind to adenine–thymine (AT)-rich regions of nuclear DNA. HMGA2 does not directly promote or inhibit the transcription of any genes, but alters the structure of DNA and promotes the assembly of protein complexes that do regulate the transcription of genes. It can be directly repressed by the well-known miRNA, lethal-7 (let-7) [141]. Sun *et al.* reported that let-7 was downregulated during the clonal expansion stage and upregulated later in the terminal differentiation stage in the adipogenic differentiation process of 3T3-L1 proadipocytes [142]. Ectopic overexpression of let-7 markedly reduced HMGA2 and blocked adipogenesis. Conversely, miR-17-92 was significantly upregulated in the clonal expansion stage and accelerated adipocyte differentiation [143]. A target gene reporter assay demonstrated that miR-17-92 directly targets the 3′ UTR region of retinoblastoma binding protein 2 (Rb2/p130), which can form heterodimers with the E2 transcription factor 4 (E2F4) and E2F5, leading to the repression of cell cycle progression [144,145]. The miRNAs regulating the MAPK signaling pathway of adipogenesis are illustrated in Figure 11.8.

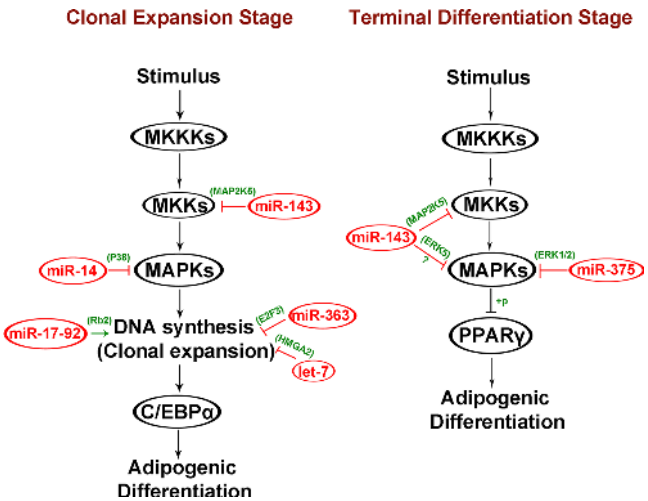



**Figure 11.8** miRNAs regulate adipogenesis by targeting the MAPK signaling pathway. The MAPK signaling pathway (MKKKs–MKKs–MAPKs) is involved in both the mitotic clonal expansion stage and the terminal differentiation stage of adipogenic differentiation. In the mitotic clonal expansion stage, DNA synthesis and cell division require the activation of the MAPK signaling pathway, whereas in the terminal differentiation stage it is the activation of MAPKs that inhibits differentiation via the phosphorylation of PPAR-γ. Thus, miRNAs targeting the MAPK signaling pathway in the mitotic clonal expansion stage may inhibit adipogenic differentiation (let-7, miR-14, miR-17-92, miR-143, and miR-363), and miRNAs targeting the MAPK signaling pathway in the terminal differentiation stage promote adipogenic differentiation (miR-375 and miR-143).

#### *11.4.3.1.5 MicroRNAs regulate adipogenesis by targeting the Wnt/β-catenin signaling pathway*

In a high-throughput microarray study, Qin *et al.* found that miR-322, miR-210, miR-194, and miR-148a might promote adipogenesis by repressing Wnt signaling, while miR-181, miR-344, and miR-27 might inhibit adipogenesis by activating Wnt signaling [146]. The targets of these miRNAs were also analyzed by bioinformatics. miR-210 was chosen as a representative miRNA to validate the predicted targets and the potential functions in adipogenesis. Mimics of miR-210 were transfected into 3T3-L1 preadipocytes and the results showed that overexpression of miR-210 stimulated lipid droplet formation, while downregulation of miR-210 strongly blocked adipogenesis, suggesting that miR-210 might play a specific role in adipogenesis. The expression of TCF 7-like 2 (T-cell specific, HMG-box) also known as TCF7L2 or TCF4, a transcription factor triggering the downstream responsive genes of Wnt signaling, was decreased. Furthermore, luciferase reporter assays showed that *TCF7L2* is the target gene of miR-210. miR-8 may be another negative regulator of Wnt signaling at multiple levels, from secretion of the ligand to the transcription of target genes. One study showed that mammalian homologues of miR-8 were capable of promoting the adipogenesis of marrow stromal cells by directly antagonizing TCF [147]. The miRNA-related Wnt/β-catenin signaling pathway in adipogenesis is illustrated in Figure 11.9.

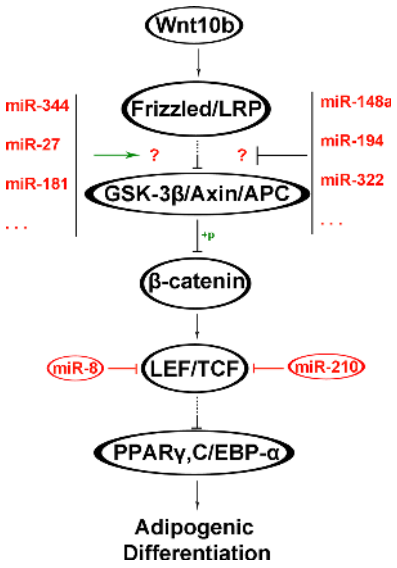



**Figure 11.9** miRNAs regulate adipogenesis by targeting the Wnt/β-catenin signaling pathway. miR-8, miR-148a, miR-194, miR-210, and miR-322 may promote adipogenesis by repressing Wnt signaling, while miR-27, miR-181, and miR-344 may inhibit adipogenesis by activating Wnt signaling. TCF, which is a transcription factor triggering the downstream genes of Wnt signaling, has been identified as the target gene of miR-210 and miR-8. The targets of the other miRNAs await identification.

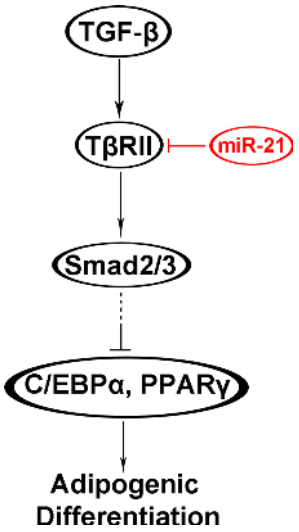



**Figure 11.10** miRNAs regulate adipogenesis by targeting the TGF-β signaling pathway. TGF-β initiates signaling by binding to TβRII in adipogenesis. Activated TβRII induces the phosphorylation of Smad2 and Smad3. Activated Smad2 and Smad3 inhibit the expression of C/EBPα and PPAR-γ. miR-21 promotes adipogenesis mainly by repressing expression of TβRII.

#### *11.4.3.1.6 MicroRNAs regulate adipogenesis by targeting the TGF-β signaling pathway*

Kim *et al.* reported that the expression level of miR-21 varied in the adipogenic differentiation process of human adipose-derived mesenchymal stromal cells. Lentiviral overexpression of miR-21 in these cells enhanced adipogenic differentiation, whereas its downregulation with AS miR-21 oligodeoxynucleotides inhibited this process. Western blot testing and the luciferase reporter assay showed that miR-21 promoted adipogenesis mainly by repressing expression of TβRII, an important member in the TGF-β signaling pathway [148]. miR-21 regulation of the TGF-β signaling pathway in adipogenesis is shown in Figure 11.10.

#### *11.4.3.1.7 MicroRNAs regulate adipogenesis by directly targeting key transcription factors*

PPARs can be targeted by miRNAs in adipogenesis. Many studies have shown that the miR-27 family is potentially an important class of negative regulators of adipogenesis. Michael *et al.* found that the expression level of miR-27b decreased during adipogenic differentiation [149]. Upregulation of miR-27b levels decreased the expression of PPAR-γ and C/EBPα. A luciferase reporter assay identified that PPAR-γ was the target gene of miR-27b. Similarly, Kim *et al.* [150] found that ectopic overexpression of another miR-27 family member, miR-27a, in 3T3-L1 preadipocytes also inhibited adipogenesis by reducing PPAR-γ expression. (Ectopic expression of a gene is the expression of a gene in an abnormal place in an organism.) Curiously, in the adipose tissue of obese mice the expression of miR-27 was upregulated and was regulated by hypoxia. Lin *et al.* found similar results during adipogenic differentiation of mouse embryonic fibroblast-derived 3T3-L1 preadipocytes and mouse bone-marrow-derived OP9 mesenchymal stem/progenitor cells [151], as did Ji *et al.* in hepatic stellate cells [152]. Martinelli *et al.* reported that miR-519d could specifically and dose dependently suppress PPAR-α protein level during adipogenic differentiation of preadipocytes [153]. The 3′ UTR of PPAR-α harbors a putative miRNA binding site that has been shown to specifically bind to miR-519d. Yang *et al.* found that miR-138 was downregulated during adipogenic differentiation of human adipose-tissue-derived stromal cells [154]. Overexpression of miR-138 effectively reduced lipid droplet accumulation. Further study identified that *EID-1*, the gene that encodes EP300-interacting inhibitor of differentiation 1, can interact with a small heterodimer partner, an endogenous enhancer of adipogenic PPAR-γ2, and is the target gene of miR-138.

C/EBPs are also targets of miRNAs in adipogenesis. Sun *et al.* reported that when the abundance of miR-31 in the murine mesenchymal stem cell line C3H10T1/2 was upregulated by induction of BMP-2, the adipogenic differentiation of BMSCs was repressed, and C/EBPα was regarded as the target gene of miR-31 [155]. Similarly, Tang *et al.* found that expression of miR-31 and miR-326 were significantly downregulated during adipogenic differentiation of adipose-derived stem cells [156]. C/EBPα was also predicted to be the target gene of the two miRNAs by bioinformatics analysis. In addition, C/EBPβ was identified as the target gene of miR-155 in the hepatic cells of C57BL/6 mice. The role of miR-155 in adipogenesis needs further study [157].

### 11.4.3.2 Regulation of adipogenesis by long noncoding RNAs

Long noncoding RNAs (lncRNAs), a significant and different class of transcripts, have been described. They were first discovered following large-scale sequencing of a full-length murine cDNA library [158]. LncRNAs are larger than 200 nucleotides and lack protein-coding function. They have been found to be transcribed throughout the eukaryotic genome. They account for the majority of the transcripts in the mammalian genome. In comparison with small noncoding RNAs, such as miRNAs, the functions of lncRNAs are as yet poorly understood [159]. However, a growing number of studies have shown that lncRNAs are involved in gene regulation at multiple levels. They play important roles in the process of embryonic development, the evolution of species, cell proliferation, cell differentiation, and diseases [160].

Sun *et al.* profiled the transcriptome of various cell models, including primary adipocytes from brown and white adipose tissue, preadipocytes, and cultured adipocytes [161]. They identified 175 lncRNAs that are specifically involved in adipogenesis. Many lncRNAs are adipose enriched, strongly induced during adipogenesis, and bound at their promoters

by crucial transcription factors such as PPAR-γ and C/EBPα. RNAi-mediated loss-of-function screens identified functional lncRNAs, and numerous lncRNAs were found to be required for functional adipogenesis. Zhao *et al.* globally profiled lncRNA gene expression during thermogenic adipocyte formation and identified brown adipose tissue lncRNA 1 (Blnc1) as a nuclear lncRNA that promoted brown and beige adipocyte differentiation and function [162]. Blnc1 formed a ribonucleoprotein complex with transcription factor early B cell factor 2 (EBF2) to stimulate the thermogenic gene program. Further, Blnc1 itself was a target of EBF2, thereby forming a feedforward regulatory loop to drive adipogenesis toward a thermogenic phenotype. (A feedforward regulatory loop involves specific circuitry in which the timely controlled expression of two genes is reciprocally regulated in order to establish correct progression of cell differentiation.)

PU.1 is an Ets family transcription factor involved in myelo-lymphoid cell differentiation but is also expressed in the adipocyte lineage. Pang *et al.* found that expression levels of PU.1 mRNA and protein in preadipocytes did not match the levels in mature adipocytes [163]. PU.1 mRNA level was higher in preadipocytes, whereas its protein was expressed in adipocytes but not in preadipocytes. They also found that miR-155 knockdown or overexpression had no effect on the levels of PU.1 mRNA and protein in preadipocytes or adipocytes. MiR-155 regulates adipogenesis not through PU.1, but via C/EBPβ, which is another target of miR-155. They also determined expression levels of PU.1 mRNA and AS (antisense) lncRNA. Compared with the level of PU.1 mRNA, the level of PU.1 AS lncRNA is much higher in preadipocytes, whereas it was the opposite in adipocytes. They further discovered that PU.1 AS lncRNA bound to its mRNA, forming an mRNA/AS lncRNA compound. The knockdown of PU.1 AS by small interfering (siRNA) inhibited adipogenesis and promoted PU.1 protein expression in both preadipocytes and adipocytes. Repression of PU.1 AS decreased the expression and secretion of adiponectin. They also found that the level of PU.1 AS lncRNA was much higher in preadipocytes than in mature adipocytes [163]. They further discovered that PU.1 AS lncRNA bound to its mRNA, forming an mRNA/AS lncRNA compound. Taken together, these findings suggest that lncRNAs play an important role in regulating adipogenesis.

## 11.5 The future

Adipogenesis is a complex physiological process that involves a large number of molecular events. The regulatory mechanisms of adipogenesis are gradually being elucidated, but many challenges remain. First, further research should be done in understanding the molecular details of the commitment of ASCs to the adipocyte lineage. Second, more signaling pathways that may be involved in adipogenesis, such as the FGF signaling pathway and the Hedgehog signaling pathway, should be studied in greater depth. Third, the epigenetic regulation of adipogenesis and the relationship between DNA/histone methylation/acetylation, miRNAs, and lncRNAs should be investigated more thoroughly. Fourth, the regulatory mechanisms of lncRNAs in adipogenesis and additional direct target genes of miRNAs need to be explored. Finally, since previous studies focused mainly on *in vitro* cell culture, more *in vivo* experiments are needed.

## References


1 M. Lean, T. Han, and J. Seidell. Impairment of health and quality of life in people with large waist circumference. *Lancet* 1998;**351**:853–856.

2 E. D. Rosen and B. M. Spiegelman. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 2006; **444**:847–853.

3 P. A. Zuk, M. Zhu, and H. Mizuno. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**:211–226.

4 D. A. De Ugarte, K. Morizono, A. Elbarbary, *et al.* Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 2003;**174**:101–109.

5 B. M. Strem, K. C. Hicok, M. Zhu, *et al.* Multipotential differentiation of adipose tissue derived stem cells. *Keio J Med* 2005;**54**: 132–141.

6 J. H. Hui, L. Li, Y. H. Teo, *et al.* Comparative study of the ability of mesenchymal stem cells derived from bone marrow, periosteum, and adipose tissue in treatment of partial growth arrest in rabbit. *Tissue Eng* 2005;**11**:904–912.

7 B. T. Mischen, K. E. Follmar, K. E. Moyer, *et al.* Metabolic and functional characterization of human adipose-derived stem cells in tissue engineering. *Plast Reconstr Surg* 2008;**122**: 725–738.

8 K. Morizono, De Ugarteda, M. Zhu, *et al.* Multilineage cells from adipose tissue gene delivery vehicles. *Hum Gene Ther* 2003;**14**: 59–66.

9 J. L. Dragoo, J. Y. Choi, J. R. Lieberman, *et al.* Bone induction by BMP-2 transduced stem cells derived from human fat. *J Orthop Res* 2003;**21**:622–629.

10 S. P. Weisberg, D. McCann, M. Desai, *et al.* Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest* 2003;**112**:1796–1808.

11 H. Xu, G. T. Barnes, Q. Yang, *et al.* Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 2003;**112**:1821–1830.

12 S. Caspar-Bauguil, B. Cousin, A. Galinier, *et al.* Adipose tissues as an ancestral immune organ: Site-specific change in obesity. *FEBS Lett* 2005;**579**:3487–3492.

13 L. Casteilla, V. Planat-Bénard, B. Cousin, *et al.* Plasticity of adipose tissue: a promising therapeutic avenue in the treatment of cardiovascular and blood diseases. *Arch Mal Coeur Vaiss* 2005;**98**: 922–926.

14 J. B. Mitchell, K. McIntosh, S. Zvonic, *et al.* Immunophenotype of human adipose-derived cells: temporal changes in stromal-associated and stem cell-associated markers. *Stem Cells* 2006;**24**: 376–385.

15 J. M. Gimble, A. J. Katz, and B. A. Bunnell. Adipose-derived stem cells for regenerative medicine. *Circ Res* 2007;**100**:1249–1260.

16 P. C. Baer and H. Geiger. Adipose-derived mesenchymal stromal/stem cells: tissue localization, characterization, and heterogeneity. *Stem Cells Int* 2012;**2012**:812693. doi: 10.1155/2012/812693

17 V. van Harmelen, T. Skurk, K. Röhrig, *et al.* Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. *Int J Obes Relat Metab Disord* 2003;**27**:889–895.

18 M. J. Oedayrajsingh-Varma, S. M. van Ham, M. Knippenberg, *et al.* Adipose tissue-derived mesenchymal stem cell yield and growth characteristics are affected by the tissue-harvesting procedure. *Cytotherapy* 2006;**8**:166–177.

19 V. van Harmelen, K. Röhrig, and H. Hauner. Comparison of proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue depot of obese subjects. *Metabolism* 2004;**53**:632–637.

20 A. Schellenberg, T. Stiehl, P. Horn, *et al.* Population dynamics of mesenchymal stromal cells during culture expansion. *Cytotherapy* 2012;**14**:401–411.

21 S. Gronthos, D. M. Franklin, H. A. Leddy, *et al.* Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* 2001;**189**:54–63.

22 M. J. O. Varma, R. G. M. Breuls, T. E. Schouten, *et al.* Phenotypical and functional characterization of freshly isolated adipose tissue-derived stem cells. *Stem Cells Dev* 2007;**16**:91–104.

23 Y. Lin, X. Chen, Z. Yan, *et al.* Multilineage differentiation of adipose-derived stromal cells from GFP transgenic mice. *Mol Cell Biochem* 2006;**285**:69–78.

24 Y. A. Romanov, A. N. Darevskaya, N. V. Merzlikina, and L. B. Buravkova. Mesenchymal stem cells from human bone marrow and adipose tissue: isolation, characterization, and differentiation potentialities. *Bull Exp Biol Med* 2005;**140**:138–143.

25 K. Timper, D. Seboek, M. Eberhardt, *et al.* Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. *Biochem Biophys Res Commun* 2006;**341**:1135–1140.

26 M. J. Seo, S. Y. Suh, Y. C. Bae, and J. S. Jung. Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 2005;**328**:258–264.

27 J. Corre, C. Barreau, B. Cousin, *et al.* Human subcutaneous adipose cells support complete differentiation but not self-renewal of hematopoietic progenitors. *J Cell Physiol* 2006;**208**: 282–288.

28 S. Gesta, Y. H. Tseng, and C. R. Kahn. Developmental origin of fat: tracking obesity to its source. *Cell* 2007;**131**:242–256.

29 N. Billon, M. C. Monteiro, and C. Dani. Developmental origin of adipocytes: new insights into a pending question. *Biol Cell* 2008;**100**:563–575.

30 B. G. Marques, D. B. Hausman, and R. J. Martin. Association of fat cell size and paracrine growth factors in development of hyperplastic obesity. *Am J Physiol* 1998;**275**:1898–1908.

31 K. Kishida, H. Kuriyama, T. Funahashi, *et al.* Aquaporin adipose, a putative glycerol channel in adipocytes. *J Biol Chem* 2000;**275**: 20896–20902.

32 Q. Q. Tang, T. C. Otto, and M. D. Lane. Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proc Natl Acad Sci U S A* 2004;**101**:9607–9611.

33 R. McBeath, D. M. Pirone, C. M. Nelson, *et al.* Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004;**6**:483–495.

34 B. Feve. Adipogenesis: cellular and molecular aspects. *Best Pract Res Clin Endocrinol* 2005;**19**:483–499.

35 E. D. Rosen and O. A. MacDougald. Adipocyte differentiation from the inside out. *Nat Rev Mol Cell Biol* 2006;**7**:885–896.

36 B. T. Porse, T. A. Pedersen, X. F. Xu, *et al.* E2F repression by C/EBPα is required for adipogenesis and granulopoiesis *in vivo*. *Cell* 2001;**107**:247–258.

37 H. Wang, P. Iakova, M. Wilde, *et al.* C/EBPα arrests cell proliferation through direct inhibition of cdk2 and cdk4. *Mol Cell* 2001;**8**:817–828.

38 L. Fajas, R. L. Landsberg, Y. H. Garcia, *et al.* E2Fs regulate adipocyte differentiation. *Dev Cell* 2002;**3**:39–49.

39 Y. M. Patel and M. D. Lane. Mitotic clonal expansion during preadipocyte differentiation: calpain-mediated turnover of p27. *J Biol Chem* 2000;**275**:17653–17660.

40 Q. Q. Tang, T. C. Otto and M. D. Lane. Mitotic clonal expansion: a synchronous process required for adipogenesis. *Proc Natl Acad Sci U S A* 2003;**100**:44–49.

41 Q. Q. Tang, T. C. Otto and M. D. Lane. CCAAT/enhancer-binding protein β is required for mitotic clonal expansion during adipogenesis. *Proc Natl Acad Sci U S A* 2003;**100**:850–855.

42 S. R. Farmer. Transcriptional control of adipocyte formation. *Cell Metab* 2006;**4**:263–273.

43 J. Font de Mora, A. Porras, N. Ahn, E. Santos. Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. *Mol Cell Biol* 1997;**17**: 6068–6075.

44 Z. L. Qiu, Y. Wei, N. Chen, *et al.* DNA synthesis and mitotic clonal expansion is not a required step for 3T3-L1 preadipocyte differentiation into adipocytes. *J Biol Chem* 2001;**276**:11988–11995.

45 A. S. Augustus, Y. Kako, H. Yagyu, and I. J. Goldberg. Routes of FA delivery to cardiac muscle: modulation of lipoprotein lipolysis alters uptake of TG-derived FA. *Am J Physiol Endocrinol Metab* 2003;**284**:331–339.

46 E. Makoveichuk, S. Castel, S. Vilaró, and G. Olivecrona. Lipoprotein lipase-dependent binding and uptake of low density lipoproteins by THP-1 monocytes and macrophages: possible involvement of lipid rafts. *Biochim Biophys Acta* 2004;**1686**:37–49.

47 J. M. Ntambi and Y. C. Kim. Adipocyte differentiation and gene expression. *J Nutr* 2000;**130**:3122–3126.

48 T. A. Yap, M. D. Garrett, M. I. Walton, *et al.* Targeting the PI3K–AKT–mTOR pathway: progress, pitfalls, and promises. *Curr Opin Pharmacol* 2008;**8**:393–412.

49 S. J. Yun, E. K. Kim, D. F. Tucker, *et al.* Isoform-specific regulation of adipocyte differentiation by Akt/protein kinase Bα. *Biochem Biophys Res Commun* 2008;**371**:138–143.

50 J. Xu and K. Liao. Protein kinase B/AKT 1 plays a pivotal role in insulin-like growth factor-1 receptor signaling induced 3T3-L1 adipocyte differentiation. *J Biol Chem* 2004;**279**:35914–35922.

51 A. Baudry, Z. Z. Yang, and B. A. Hemmings. PKBα is required for adipose differentiation of mouse embryonic fibroblasts. *J Cell Sci* 2006;**119**:889–897.

52 X. D. Peng, P. Z. Xu, M. L. Chen, *et al.* Dwarfism, impaired skin development, skeletal muscle atrophy, delayed bone development, and impeded adipogenesis in mice lacking Akt1 and Akt2. *Genes Dev* 2003;**17**:1352–1365.

53 H. J. Park, B. Y. Chung, M. K. Lee, *et al.* Centipede grass exerts anti-adipogenic activity through inhibition of C/EBPβ, C/EBPα, and PPARγ expression and the AKT signaling pathway in 3T3-L1 adipocytes. *BMC Complement Altern Med* 2012;**12**:230.

54 J. E. B. Reusch, L. Colton, and D. J. Klemm. CREB activation induces adipogenesis in 3T3-L1 cells. *Mol Cell Biol* 2000;**20**: 1008–1020.

55 J. W. Zhang, D. J. Klemm, C. Vinson, and M. D. Lane. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein β gene during adipogenesis. *J Biol Chem* 2004;**279**:4471–4478.

56 F. Chang, L. S. Steelman, J. G. Shelton, *et al.* Regulation of cell cycle progression and apoptosis by the Ras/Raf/MEK/ERK pathway. *Int J Oncol* 2003;**22**:469–480.

57 A. M. Manning and R. J. Davis. Targeting JNK for therapeutic benefit: from junk to gold? *Nat Rev Drug Discov* 2003;**2**:554–565.

58 E. Hu, J. B. Kim, P. Sarraf, and B. M. Spiegelman. Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. *Science* 1996;**274**:2100–2103.

59 K. A. Burns and J. P. Vanden Heuvel. Modulation of PPAR activity via phosphorylation. *Biochim Biophys Acta* 2007;**1771**:952–960.

60 F. Bost, M. Aouadi, L. Caron, and B. Binétruy. The role of MAPKs in adipocyte differentiation and obesity. *Biochimie* 2005;**87**:51–56.

61 M. D. Gordon and R. Nusse. Wnt signaling: multiple pathways, multiple receptors, and multiple transcription factors. *J Biol Chem* 2006;**281**:22429–22433.

62 C. Christodoulides, C. Lagathu, J. K. Sethi, and A. Vidal-Puig. Adipogenesis and WNT signalling. *Trends Endocrinol Metab* 2009;**20**:16–24.

63 M. J. Koslowski, I,. Kübler, M. Chamaillard, *et al.* Genetic variants of Wnt transcription factor TCF-4 (TCF7L2) putative promoter region are associated with small intestinal Crohn's disease. *PLoS ONE* 2009;**4**:e4496.

64 S. E. Ross, N. Hemati, K. A. Longo, *et al.* Inhibition of adipogenesis by Wnt signaling. *Science* 2000;**289**:950–953.

65 J. Massagué. How cells read TGF-β signals. *Nat Rev Mol Cell Biol* 2000;**1**:169–178.

66 B. P. Sullivan, K. M. Kassel, S. Manley, *et al.* Regulation of transforming growth factor-β1-dependent integrin β6 expression by p38 mitogen-activated protein kinase in bile duct epithelial cells. *J Pharmacol Exp Ther* 2011;**337**:471–478.

67 K. Terai, M. K. Call, H. Liu, *et al.* Crosstalk between TGF-β and MAPK signaling during corneal wound healing. *Invest Ophthalmol Vis Sci* 2011;**52**:8208–8215.

68 R. A. Ignotz and J. Massague. Type β transforming growth factor controls the adipogenic differentiation of 3T3 fibroblasts. *Proc Natl Acad Sci U S A* 1985;**82**:8530–8534.

69 R. L. Sparks and R. E. Scott. Transforming growth factor type β is a specific inhibitor of 3T3T mesenchymal stem cell differentiation. *Exp Cell Res* 1986;**165**:345–352.

70 F. M. Torti, S. V. Torti, J. W. Larrick, and G. M. Ringold. Modulation of adipocyte differentiation by tumor necrosis factor and transforming growth factor β. *J Cell Biol* 1989;**108**: 1105–1113.

71 D. E. Clouthier, S. A. Comerford, and R. E. Hammer. Hepatic fibrosis, glomerulosclerosis, and a lipodystrophy-like syndrome in PEPCK–TGF-β1 transgenic mice. *J Clin Invest* 1997;**100**: 2697–2713.

72 J. M. Lehmann, L. B. Moore, T. A. Smith-Oliver, *et al.* An anti-diabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). *J Biol Chem* 1995;**270**:12953–12956.

73 R. Brandes, R. Arad, and J. Bar-Tana. Inducers of adipose conversion activate transcription promoted by a peroxisome proliferators response element in 3T3-L1 cells. *Biochem Pharmacol* 1995;**50**: 1949–1951.

74 A. Chawla and M. A. Lazar. Peroxisome proliferator and retinoid signaling pathways co-regulate preadipocyte phenotype and survival. *Proc Natl Acad Sci U S A* 1994;**91**:1786–1790.

75 A. Ibrahimi, L. Teboul, D. Gaillard, *et al.* Evidence for a common mechanism of action for fatty acids and thiazolidinedione anti-diabetic agents on gene expression in preadipose cells. *Mol Pharmacol* 1994;**46**:1070–1076.

76 L. Teboul, D. Gaillard, L. Staccini, *et al.* Thiazolidinediones and fatty acids convert myogenic cells into adipose-like cells. *J Biol Chem* 1995;**270**:28183–28187.

77 A. Chawla, J. J. Repa, R. M. Evans, and D. J. Mangelsdorf. Nuclear receptors and lipid physiology: opening the X-files. *Science* 2001;**294**:1866–1870.

78 M. Seimandi, G. Lemaire, A. Pillon, *et al.* Differential responses of PPARα, PPARδ, and PPARγ reporter cell lines to selective PPAR synthetic ligands. *Anal Biochem* 2005;**344**:8–15.

79 D. K. Krämer, M. Ahlsén, J. Norrbom, *et al.* Human skeletal muscle fibre type variations correlate with PPARα, PPARδ and PGC-1α mRNA. *Acta Physiol* 2006;**188**:207–216.

80 S. A. Kliewer, B. M. Forman, B. Blumberg, *et al.* Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A* 1994;**91**: 7355–7359.

81 O. Braissant, F. Foufelle, C. Scotto, *et al.* Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-α, -β, and -γ in the adult rat. *Endocrinolgy* 1996;**137**:354–366.

82 M. Lehrke and M. A. Lazar. The many faces of PPARγ. *Cell* 2005;**123**:993–999.

83 P. Shen, M. H. Liu, T. Y. Ng, *et al.* Differential effects of isoflavones, from *Astragalus membranaceus* and *Pueraria thomsonii,* on the activation of PPARα, PPARγ, and adipocyte differentiation *in vitro*. *J Nutr* 2006;**136**:899–905.

84 A. Mazzucotelli, N. Viguerie, C. Tiraby, *et al.* The transcriptional coactivator peroxisome proliferator-activated receptor (PPAR)γ coactivator-1α and the nuclear receptor PPARα control the expression of glycerol kinase and metabolism genes independently of PPARγ activation in human white adipocytes. *Diabetes* 2007;**56**: 2467–2475.

85 N. D. Wang, M. J. Finegold, A. Bradley, *et al.* Impaired energy homeostasis in C/EBP alpha knockout mice. *Science* 1995;**269**: 1108–1112.

86 R. J. Christy, K. H. Kaestner, D. E. Geiman, and M. D. Lane. CCAAT/enhancer binding protein gene promoter: binding of nuclear factors during differentiation of 3T3-L1 preadipocytes. *Proc Natl Acad Sci U S A* 1991;**88**:2593–2597.

87 F. T. Lin, O. A. MacDougald, A. M. Diehl, and M. D. Lane. A 30-kDa alternative translation product of the CCAAT/enhancer binding protein alpha message: transcriptional activator lacking antimitotic activity. *Proc Natl Acad Sci U S A* 1993;**90**: 9606–9610.

88 O. A. MacDougald and M. D. Lane. Adipocyte differentiation. When precursors are also regulators. *Curr Biol* 1995;**5**: 618–621.

89 Z. Wu, N. L. R. Bucher, and S. R. Farmer. Induction of peroxisome proliferator-activated receptor γ during the conversion of 3T3 fibroblasts into adipocytes is mediated by C/EBPβ, C/EBPδ, and glucocorticoids. *Mol Cell Biol* 1996;**16**:4128–4136.

90 I. Shimomura, H. Shimano, B. S. Kom, *et al*. Nuclear sterol regulatory element-binding proteins activate genes responsible for the entire program of unsaturated fatty acid biosynthesis in transgenic mouse liver. *J Biol Chem* 1998;**273**:35299–35306.

91 J. B. Kim and B. M. Spiegelman. ADD1/SREBP1 promotes adipocyte differentiation and gene expression linked to fatty acid metabolism. *Gene Dev* 1996;**10**:1096–1107.

92 J. B. Kim, H. M. Wright, M. Wright, and B. M. Spiegelman. ADD1/SREBP1 activates PPARγ through the production of endogenous ligand. *Proc Natl Acad Sci U S A* 1998;**95**:4333–4337.

93 I. R. Henderson and S. E. Jacobsen. Epigenetic inheritance in plants. *Nature* 2007;**447**:418–424.

94 R. Barres and J. R. Zierath. DNA methylation in metabolic disorders. *Am J Clin Nutr* 2011;**93**:897–900.

95 A. C. D'Alessio, I. C. Weaver, and M. Szyf. Acetylation-induced transcription is required for active DNA demethylation in methylation-silenced genes. *Mol Cell Biol* 2007;**27**:7462–7474.

96 J. G. Zhu, L. Xia, C. B. Ji, *et al*. Differential DNA methylation status between human preadipocytes and mature adipocytes. *Cell Biochem Biophys* 2012;**63**:1–15.

97 H. X. Li, L. Xiao, C. Wang, *et al*. Review: epigenetic regulation of adipocyte differentiation and adipogenesis. *J Zhejiang Univ Sci B* 2010;**11**:784–791.

98 A. Noer, A. L. Sorensen, A. C. Boquest, and P. Collas. Stable CpG hypomethylation of adipogenic promoters in freshly isolated, cultured, and differentiated mesenchymal stem cells from adipose tissue. *Mol Biol Cell* 2006;**17**:3543–3556.

99 K. Fujiki, F. Kano, K. Shiota, and M. Murata. Expression of the peroxisome proliferator activated receptor γ gene is repressed by DNA methylation in visceral adipose tissue of mouse models of diabetes. *BMC Biol* 2009;**7**:38.

100 R. J. Klose and A. P. Bird. Genomic DNA methylation: the mark and its mediators. *Trends Biochem Sci* 2006;**31**:89–97.

101 N. Salma, H. Xiao, E. Mueller, and A. N. Imbalzano. Temporal recruitment of transcription factors and SWI/SNF chromatin remodeling enzymes during adipogenic induction of the peroxisome proliferator-activated receptor γ nuclear hormone receptor. *Mol Cell Biol* 2004;**24**:4651–4663.

102 Y. Okada, H. Sakaue, T. Nagare, and M. Kasuga. Diet-induced up-regulation of gene expression in adipocytes without changes in DNA methylation. *Kobe J Med Sci* 2009;**54**:241–249.

103 I. Melzner, V. Scott, K. Dorsch, *et al*. Leptin gene expression in human preadipocytes is switched on by maturation-induced demethylation of distinct CpGs in its proximal promoter. *J Biol Chem* 2002;**277**:45420–45427.

104 M. Kussmann, L. Krause, and W. Siffert. Nutrigenomics: where are we with genetic and epigenetic markers for disposition and susceptibility. *Nat Rev* 2010;**68**:38–47.

105 F. I. Milagro, J. Campión, D. F. García-Díaz, *et al*. High fat diet-induced obesity modifies the methylation pattern of leptin promoter in rats. *J Physiol Biochem* 2009;**65**:1–9.

106 Y. Wang, H. Suzuki, T. Yokoo, *et al*. WGEF is a novel RhoGEF expressed in intestine, liver, heart,and kidney. *Biochem Biophys Res Commun* 2004;**324**:1053–1058.

107 M. Nethe and P. L. Hordijk. The role of ubiquitylation and degradation in RhoGTPase signalling. *J Cell Sci* 2010;**123**: 4011–4018.

108 R. Barres and J. R. Zierath. DNA methylation in metabolic disorders. *Am J Clin Nutr* 2011;**93**:897–900.

109 B. D. Strahl and C. D. Allis. The language of covalent histone modifications. *Nature* 2000;**403**:41–45.

110 T. Jenuwein and C. D. Allis. Translating the histone code. *Science* 2001;**293**:10741080

111 S. N. Kim, H. Y. Choi, and Y. K. Kim. Regulation of adipocyte differentiation by histone deacetylase inhibitors. *Arch Pharm Res* 2009;**32**:535–541.

112 T. K. Chatterjee, G. Idelman, V. Blanco, *et al*. Histone deacetylase 9 is a negative regulator of adipogenic differentiation. *J Biol Chem* 2011;**286**:27836–27847.

113 E. J. Yoo, J. J. Chung, S. S. Choe, *et al*. Down-regulation of histone deacetylases stimulates adipocyte differentiation. *J Biol Chem* 2006;**281**:6608–6615.

114 Y. Zhu, C. Qi, J.R. Korenberg, *et al*. Structural organization of mouse peroxisome proliferator-activated receptor gamma (mPPARγ) gene: alternative promoter use and different splicing yield two mPPARγ isoforms. *Proc Natl Acad Sci U S A* 1995;**92**: 7921–7925.

115 T.S. Mikkelsen, Z. Xu, X. Zhang, *et al*. Comparative epigenomic analysis of murine and human adipogenesis. *Cell* 2010;**143**: 156–169.

116 L. Wang, Q. Jin, J. E. Lee, *et al*. Histone H3K27 methyltransferase Ezh2 represses Wnt genes to facilitate adipogenesis. *Proc Natl Acad Sci U S A* 2010;**107**:7317–7322.

117 P. D. Zarnore and B. Haley. The big world of small RNAs. *Science* 2005;**309**:1519–1524.

118 D. P. Bartel. MicroRNAs: target recognition and regulatory functions. *Cell* 2009;**136**:215–233.

119 S. Vasudevan and J. A. Steitz. AU-rich-element-mediated up-regulation of translation by FXR1 and argonaute 2. *Cell* 2007;**128**:1105–1118.

120 S. Vasudevan, Y. Tong, and J. A. Steitz. Switching from repression to activation: microRNAs can up-regulate translation. *Science* 2007;**318**:1931–1934.

121 Y. Lee, K. Jeon, J. T. Lee, *et al*. MicroRNA maturation: stepwise processing and subcellular localization. *EMBO J* 2002;**21**: 4663–4670.

122 V. Ambros, R. C. Lee, A. Lavanway, *et al*. MicroRNAs and other tiny endogenous RNAs in *C. elegans*. *Curr Biol* 2003;**13**: 807–818.

123 S. L. Lin, D. Chang, D. Y. Wu, and S. Y. Ying. A novel RNA splicing-mediated gene silencing mechanism potential for genome evolution. *Biochem Biophys Res Commun* 2003;**310**:754–760.

124 A. Rodriguez, S. Griffiths-Jones, J. L. Ashurst, and A. Bradley. Identification of mammalian microRNA host genes and transcription units. *Genome Res* 2004;**14**:1902–1910.

125 D. P. Bartel. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;**116**:281–297.

126 A. B. He, L. L. Zhu, N. Gupta, *et al.* Overexpression of micro ribonucleic acid 29, highly up-regulated in diabetic rats, leads to insulin resistance in 3T3-L1 adipocytes. *Mol Endocrinol* 2007;**21**:2785–2794.

127 A. E. Ouaamari, N. Baroukh, G. A. Martens, *et al.* miR-375 targets 3′-phosphoinositide dependent protein kinase-1 and regulates glucose-induced biological responses in pancreatic β-cells. *Diabetes* 2008;**57**:2708–2717.

128 H. Y. Ling, H. S. Ou, S. D. Feng, *et al.* Changes in microRNA (miR) profile and effects of miR-320 in insulin-resistant 3T3-L1 adipocytes. *Clin Exp Pharmacol Physiol* 2009;**36**:32–39.

129 C. Yi, W. D. Xie, F. Lia, *et al.* MiR-143 enhances adipogenic differentiation of 3T3-L1 cells through targeting the coding region of mouse pleiotrophin. *FEBS Lett* 2011;**585**:3303–3309.

130 S. D. Jordan, M. Krüger, D. M. Willmes, *et al.* Obesity-induced overexpression of miRNA-143 inhibits insulin-stimulated AKT activation and impairs glucose metabolism. *Nat Cell Biol* 2011;**13**:434–446.

131 P. Rajasethupathy, F. Fiumara, R. Sheridan, *et al.* Characterization of small RNAs in Aplysia reveals a role for miR-124 in constraining synaptic plasticity through CREB. *Neuron* 2009;**63**:803–817.

132 M. J. Millan. MicroRNA in the regulation and expression of serotonergic transmission in the brain and other tissues. *Curr Opin Pharmacol* 2011;**11**:11–22.

133 S. H. Liu, Y. Yang, and J. R. Wu. TNFα-induced up-regulation of miR-155 inhibits adipogenesis by down-regulating early adipogenic transcription factors. *Biochem Biophys Res Commun* 2011;**414**:618–624.

134 N. Klöting, S. Berthold, P. Kovacs, *et al.* MicroRNA expression in human omental and subcutaneous adipose tissue. *PLoS ONE* 2009;**4**:e4699.

135 C. Esau, X. L. Kang, E. Peralta, *et al.* MicroRNA-143 regulates adipocyte differentiation. *J Biol Chem* 2004;**279**: 52361–52365.

136 R. Takanabe, K. Ono, Y. Abe, *et al.* Up-regulated expression of microRNA-143 in association with obesity in adipose tissue of mice fed high-fat diet. *Biochem Biophys Res Commun* 2008;**376**: 728–732.

137 A. Z. Oskowitz, J. Lu, P. Penfornis, *et al.* Human multipotent stromal cells from bone marrow and microRNA: regulation of differentiation and leukemia inhibitory factor expression. *Proc Natl Acad Sci U S A* 2008;**105**:18372–18377.

138 L Chen, C Li, J. H. Cui, *et al.* MicroRNA-143 regulates adipogenesis by modulating the MAP2K5-ERK5 signaling. *Sci Rep* 2014;**4**: 3819.

139 H. Y. Ling, G. B. Wen, S. D. Feng, *et al.* MicroRNA-375 promotes 3T3-L1 adipocyte differentiation through modulation of extracellular signal-regulated kinase signaling. *Clin Exp Pharmacol Physiol* 2011;**38**:239–246.

140 P. Z. Xu, S. Y. Vernooy, M. Guo, and B. A. Hay. The Drosophila microRNA mir-14 suppresses cell death and is required for normal fat metabolism. *Curr Biol* 2003;**13**:790–795.

141 C. Mayr, M. T. Hemann, and D. P. Bartel. Disrupting the pairing between let-7 and Hmga2 enhances oncogenic transformation. *Science* 2007;**315**:1576–1579.

142 T. Sun, M. Fu, A. L. Bookout, *et al.* MicroRNA let-7 regulates 3T3-L1 adipogenesis. *Mol Endocrinol* 2009;**23**:925–931.

143 Q. Wang, Y. C. Li, J. Wang, *et al.* miR-17-92 cluster accelerates adipocyte differentiation by negatively regulating tumor suppressor Rb2/p130. *Proc Natl Acad Sci U S A* 2008;**105**:2889–2894.

144 D. Cobrinik, P. Whyte, D. S. Peeper, *et al.* Cell cycle-specific association of E2F with the p130 E1A-binding protein. *Genes Dev* 1993;**7**:2392–2404.

145 N. Dyson. The regulation of E2F by pRB-family proteins. *Genes Dev* 1998;**12**:2245–2262.

146 L. Qin, Y. Chen, Y. Niu, *et al.* A deep investigation into the adipogenesis mechanism: profile of microRNAs regulating adipogenesis by modulating the canonical Wnt/β-catenin signaling pathway. *BMC Genomics* 2010;**11**:320.

147 J. A. Kennell, I. Gerin, O. A. MacDougald, and K. M. Cadigan. The microRNA miR-8 is a conserved negative regulator of Wnt signaling. *Proc Natl Acad Sci U S A* 2008;**105**: 15417–15422.

148 Y. J. Kim, S. J. Hwang, Y. C. Bae, and J. S. Jung. MiR-21 regulates adipogenic differentiation through the modulation of TGF-beta signaling in mesenchymal stem cells derived from human adipose tissue. *Stem Cells* 2009;**27**:3093–3102.

149 K. Michael, C. Fischer, S. Nowitsch, *et al.* MicroRNA miR-27b impairs human adipocyte differentiation and targets PPARγ. *Biochem Biophys Res Commun* 2009;**390**:247–251.

150 S. Y. Kim, A. Y. Kim, H. W. Lee, *et al.* miR-27a is a negative regulator of adipocyte differentiation via suppressing PPARγ expression. *Biochem Biophys Res Commun* 2010;**392**:323–328.

151 Q. Lin, Z. Gao, R. M. Alarcon, *et al.* A role of miR-27 in the regulation of adipogenesis. *FEBS J* 2009;**276**:2348–2358.

152 J. L. Ji, J. S. Zhang, G. C. Huang, *et al.* Over-expressed microRNA-27a and 27b influence fat accumulation and cell proliferation during rat hepatic stellate cell activation. *FEBS Lett* 2009;**583**: 759–766.

153 R. Martinelli, C. Nardelli, V. Pilone, *et al.* miR-519d overexpression is associated with human obesity. *Obesity* 2010;**18**: 2170–2176.

154 Z. Yang, C. Bian, H. Zhou, *et al.* MicroRNA hsa-miR-138 inhibits adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells through adenovirus EID-1. *Stem Cells Dev* 2011;**20**:259–267.

155 F. Sun, J. Wang, Q. Pan, *et al.* Characterization of function and regulation of miR-24-1 and miR-31. *Biochem Biophys Res Commun* 2009;**380**:660–665.

156 Y. F. Tang, Y. Zhang X. Y. Li, *et al.* Expression of miR-31, miR-125b-5p, and miR-326 in the adipogenic differentiation process of adipose-derived stem cells. *OMICS* 2009;**13**:331–336.

157 B. Wang, S. Majumder, G. Nuovo, *et al.* Role of microRNA-155 at early stages of hepatocarcinogenesis induced by choline-deficient and amino acid-defined diet in C57BL/6 mice. *Hepatology* 2009;**50**:1152–1161.

158 Y. Okazaki, M. Furuno, T. Kasukawa, *et al.* Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. *Nature* 2002;**420**:563–573.

159 P. Kapranov, J. Cheng, S. Dike, *et al*. RNA maps reveal new RNA classes and a possible function for pervasive transcription. *Science* 2007;**316**:1484–1488.

160 T. R. Mercer, M. E. Dinger, and J. S. Mattick. Long non-coding RNAs: insights into functions. *Nat Rev Genet* 2009;**10**:155–159.

161 L. Sun, L. A. Goff, C. Trapnell, *et al*. Long noncoding RNAs regulate adipogenesis. *Proc Natl Acad Sci U S A* 2013;**110**:3387–3392.

162 X. Y. Zhao, S. Li, and G. X. Wang. Long noncoding RNA transcriptional regulatory circuit drives thermogenic adipocyte differentiation. *Mol Cell* 2014;**55**:372–382.

163 W. J. Pang, L. G. Lin, Y. Xiong, *et al*. Knockdown of PU.1 AS lncRNA inhibits adipogenesis through enhancing PU.1 mRNA translation. *J Cell Biochem* 2013;**114**:2500–2512.

CHAPTER 12

# Modulation of osteogenic differentiation in mesenchymal stromal cells

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## 12.1 Introduction

The late 1990s were marked by the significant emergence of pluripotent stem cell lines derived from human embryos and essentially the nascence of regenerative medicine [1]. At the time it was hoped that stem cell therapy would provide an unprecedented opportunity of curing millions of patients suffering from debilitating diseases. However, unresolved ethical issues relating to the origin of the cells generated major legal, ethical, and political obstacles that remain to date. In the first decade of the 21st century, induced pluripotent stem cells were described; these did not have the ethical issues associated with human embryonic stem cells, but both shared, by definition, the ability to form teratomas, and the technology of differentiating functional end cells such as neurons or cardiomyocytes remains challenging.

Adult stem cells, such as bone- or bone marrow-derived mesenchymal stromal cells (MSCs), are considered an ethically and morally acceptable alternative to embryonic stem cells and induced pluripotent stem cells. While initially thought to be more restricted in their potential applications, the identification and characterization of MSCs in an increasing number of tissues has led to a surge of research and clinical interest in utilizing these cells for cell-based regenerative therapies [2,3]. Furthermore, clarification on the multifaceted mechanisms by which these cells exert their effects has allowed better understanding of the modulation of stem cell activity both *in vitro* and *in vivo* [4].

True mesenchymal stem cells that fulfill the criteria of both self-renewal and differentiation to functional end cells have been described. However, mesenchymal stem cells are rare cells and therefore their progeny - undifferentiated mesenchymal stromal (or progenitor) cells – are isolated by expansion in *ex vivo* culture for most laboratory investigations and for clinical trials. The abbreviation MSCs will refer to mesenchymal stromal cells in this chapter unless specifically indicated otherwise.

Bone regeneration is an area that may benefit from a cell-based approach [5]. Each year, millions of patients undergo treatment for bone fractures, with most healing satisfactorily using current orthopedic techniques. Nevertheless, fracture nonunion and bone defects remain a significant clinical challenge in a minority of such cases, especially when associated with diseases such as diabetes, osteoarthritis, and osteoporosis [6]. While several studies have shown efficacy of cell-based therapy for bone regeneration, many issues remain unresolved. For instance, a clear understanding of the molecular mechanisms governing

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osteogenic differentiation of MSCs *in vivo* remains to be established. More recently, better insights into the optimal sources of MSCs that demonstrate osteogenic propensity and how these contribute to tissue repair *in vivo* are emerging. Finally, the ability to monitor implanted cells to assess where they go and what they do when they arrive there is necessary for further development of successful therapies. Here, we explore these issues and examine the success to date of MSCs in the context of a cellular therapy in bone-related orthopedic disorders.

## 12.2 Biology

### 12.2.1 Sources of mesenchymal stromal cells

Mesenchymal stem/stromal cells were first described by Friedenstien *et al.* as plastic-adherent cells capable of differentiation towards the osteogenic lineage [7]. Subsequently, the ability of MSCs to differentiate towards the chondrogenic lineage was shown, identifying a role of the cells in cartilage as well as endochondral bone formation [8,9]. Similarly, the osteogenic differentiation of MSCs facilitates direct bone formation via intramembranous ossification. This ability to form bone via multiple lineages has led to extensive research into these cells for tissue engineering applications. Since their discovery in bone marrow, MSCs have been identified in virtually every tissue within the body, including adipose tissue and placenta [10–14]. Interestingly, the location of MSCs within each tissue seems to affect their physiological function and differentiation capabilities. Nowhere is this more evident than within bone and bone marrow.

MSCs in bone and bone marrow exist within a number of anatomical locations. Two distinct niches exist in bone marrow: the perivascular niche where MSC precursors known as pericytes exist in association with blood vessels [15], and the endosteal or bone-lining niche, where MSCs are found on the surface of bone [16–18]. MSC progeny such as osteoblasts and fibroblasts are found at the endosteal niche [18]. Additionally, lining the surface of bone is a thin layer of connective tissue known as the periosteum that has also been identified as a source of MSCs [19–21]. It is unclear which endogenous cell population contributes toward tissue repair after fractures with no evidence to date definitively identifying the population of MSCs responsible for bone repair. A comprehensive review on the healing of postnatal fractures by Gerstenfeld *et al.* highlighted this issue [22]. During fracture healing, bone repair is initiated in distinct crescent-shaped cartilaginous centers. Two centers develop symmetrically to the fracture line with intramembranous bone formation occurring concurrently at separate centers proximally and distally. These two distinct bone healing responses initiated upon injury may be induced by different cells located in the surrounding tissue. The bone marrow itself and the surrounding periosteum and soft tissue may all be sources of these reparative cells [22]. The periosteum is believed to be the primary source of cells required to form the callus [23], with removal of the periosteum resulting in incomplete fracture callus formation [22]. The periosteum has also been proposed as a novel source of progenitor cells capable of contributing to bone regeneration [21]. In a direct comparison, periosteum-derived rat MSCs produced 100-fold more colonies than bone marrow-derived MSCs and had a higher proliferative rate and trilineage differentiation potential [20]. Periosteal cells also maintained chondrogenic differentiation potential *in vitro* after prolonged culture [24]. Human studies on the periosteum as a source of such cells has also identified this tissue as being superior to other sites in terms of *in vitro* differentiation and proliferation potential [25]. Skeletal muscle has also been explored as a potential source of osteoprogenitors that contribute to bone formation. Ectopic bone formation can occur within muscle [26], and a subpopulation of muscle progenitor cells, identified by expression of alkaline phosphatase (ALP) after exposure to bone morphogenetic protein (BMP)-2, was observed in the lacunae of newly formed bone matrix when injected into the hindlimbs of mice with severe congenital immune deficiency. These cells are co-localized with osteocalcin (OC)-producing cells, indicating potential differentiation to osteoblasts and osteocytes [26].

However, identification of a definitive cell source for endochondral ossification in fracture callus has not been described. A number of MSC populations within the bone marrow that may contribute to bone repair have been identified based on expression of surface markers. Sacchetti *et al.* identified a population of osteoprogenitors based on expression of CD146 (also known as melanoma cell adhesion molecule) [17]. These cells were deemed to be *bona fide* mesenchymal stem cells after subcutaneous transplantation into nude (athymic immune deficient) mice resulted in formation of bone and induced the hematopoietic stem cell niche [17]. Prior to this, CD271, also known as low-affinity nerve growth factor receptor, was shown to select for the entire fibroblastic colony-forming unit (CFU-F) population within bone marrow, thus identifying the MSC fraction [27]. $CD271^+$ cells were subsequently shown to reside in the intramedullary cavity of long bones [28]. Combining both CD146 and CD271 expression resulted in the identification of the two distinct MSC niches already described: the endosteal niche and the perivascular niche, with $CD271^+$ $CD146^+$ cells residing in the perivascular niche and $CD271^+$ $CD146^-$ residing in the endosteal niche [29]. The presence of MSCs at a bone-lining niche and their osteogenic potential suggest a role for these cells in maintaining bone homeostasis (and see Chapter 32).

### 12.2.2 Cellular regulation of osteogenic differentiation from mesenchymal stromal cells

Bone is a highly dynamic tissue composed of type I collagen deposited with hydroxyapatite, also known as calcium phosphate. This provides the framework of the highly cellularized, highly vascularized bone tissue. It has been reported that approximately 10% of bone in adult vertebrates is remodeled every year [30]. For normal bone homeostasis this process must be highly regulated, with dysregulation resulting in catabolic bone

diseases such as osteoporosis and anabolic bone diseases such as osteopetrosis [31]. The three main cellular components involved in skeletogenesis and bone homeostasis are osteocytes and osteoblasts, which differentiate from MSCs, and osteoclasts derived from a hematopoietic stem cell lineage [32,33]. There are three modes of communication between osteoblasts and osteoclasts as outlined by Matsuo and Irie [34]. The first occurs via direct contact, whereby membrane-bound ligands interact with receptors, most notably the receptor activator of nuclear factor kappa-B and its ligand (RANK/RANKL). The second is the formation of gap junctions mediated by connexin 43 between the two cells, which allows passage of water-soluble molecules [35,36]. The third method of communication is the production and recognition of paracrine factors such as sclerostin, a potent inhibitor of bone formation [37]. Immune cells have also been reported to mediate bone homeostasis, which will be discussed later [38,39].

Coupling of osteoblasts to produce new bone and osteoclasts to resorb bone is essential for maintenance of healthy bone, and this process is dependent on RANK, its ligand RANKL, and the decoy receptor osteoprotegerin (OPG) (Figure 12.1) [40].

Of the three molecules, OPG was the first to be discovered owing to its inhibitory effect on osteoclast development both *in vitro* and *in vivo* [41,42]. As a consequence, it was initially termed osteoclastogenesis inhibitory factor (OCIF) before being rebranded as OPG, meaning "protector of bone" [43]. Concurrently, members of the tumor necrosis factor (TNF) cytokine family were described; TNF-related activation-induced cytokine (TRANCE), now commonly referred to as RANK [44] and its ligand RANKL, was also identified by the same group as a highly upregulated molecule in T cells after antigen stimulation [43].

One of the key functions of osteoblasts, besides induction of mineralization of bone extracellular matrix, is induction of osteoclast differentiation [45]. This process is highly regulated, with the immune system being a key player. The existence of the relationship between the immune system and bone has been known for some time and highlights the complexity involved in maintenance of bone homeostasis. Studies carried out nearly four decades ago reported the ability of stimulated immune cells to produce soluble factors capable of inducing osteoclast-mediated bone resorption [46]. Since then, the role of the immune system in bone homeostasis has been widely reported, particularly as similar pathways are activated during development of both systems [47]. In bone, RANKL is produced by osteoblasts and is fundamental to osteoclast activation and differentiation [40]. Apart from osteoblasts, a number of immune cells also produce RANK, including B cells, T cells, monocytes, neutrophils, and dendritic cells [38,39]. This is a key mechanism of immune activation of osteoclast differentiation and results in increased bone resorption. Furthermore, these immune cells can also produce proinflammatory cytokines that can lead to bone damage, adding to the bone loss effect that occurs through the RANK/RANKL pathway [48,49]. In mouse models, disruption of either RANK or RANKL results in mice presenting with an osteopetrotic ("stony bone" or marble bone disease) phenotype and tooth defects due to a lack of osteoclasts to negatively regulate bone [50]. Conversely, mice lacking OPG present with advanced osteoporosis due to the increase in osteoclast number and activity [50]. A number of disease states have been identified in humans due to mutations in the RANK/RANKL system, including Paget's disease of bone, which occurs as a result of mutated OPG [51], familial expansile osteolysis due to mutations in RANK [52], and autosomal recessive osteopetrosis due to RANKL mutations [53].

Stimulation of RANKL in osteoblasts can be regulated by a number of factors, which include parathyroid hormone (PTH), PTH-related protein (PTHrP), 1,25-$(OH)_2$-vitamin-$D_3$, TNF-α, and interleukins (ILs) such as IL-8 and IL-17 [54–57]. Conversely, attenuation of RANKL expression can be regulated by transforming growth factor β (TGF-β) and estrogens [57]. As a result, this pathway has been a therapeutic target for drug development for bone loss diseases such as osteoporosis (Figure 12.2). Similarly, estrogen-dependent RANKL expression can account for the high prevalence of osteoporosis in postmenopausal women with reduced estrogen levels. The incidence of osteoporosis in those over the age of 50 is three to four times higher in women than men [58]. This is due primarily to the decrease in estrogen levels postmenopause [59]. Reduced estrogen levels lead to reduced OPG expression, shifting normal bone

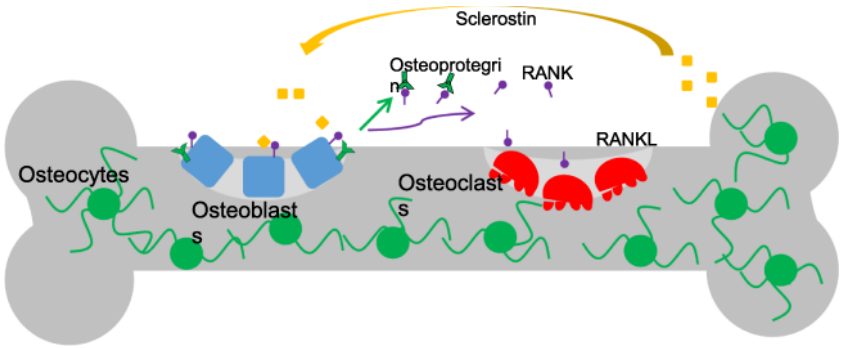



**Figure 12.1** RANK/RANKL/OPG interaction in osteoblast/osteoclast communication. Bone-lining osteoblasts produce RANK, which can bind to RANKL on osteoclasts. Osteoblasts also produce decoy receptor OPG, which can dampen the effect of RANK on osteoclasts. As osteoblasts get embedded in new bone, they terminally differentiate into osteocytes, which secrete sclerostin through their dendrites, which can inhibit the action of osteoblasts. (See color plate section.)



**Figure 12.2** Osteoporosis. Lateral spine X-ray showing wedge fractures of lumbar vertebrae 1 and 2 due to severe osteoporosis. *Source*: http://commons.wikimedia.org/wiki/File:L1_2_vertebral_fracture.jpg#mediaviewer/File:L1_2_vertebral_fracture.jpg.

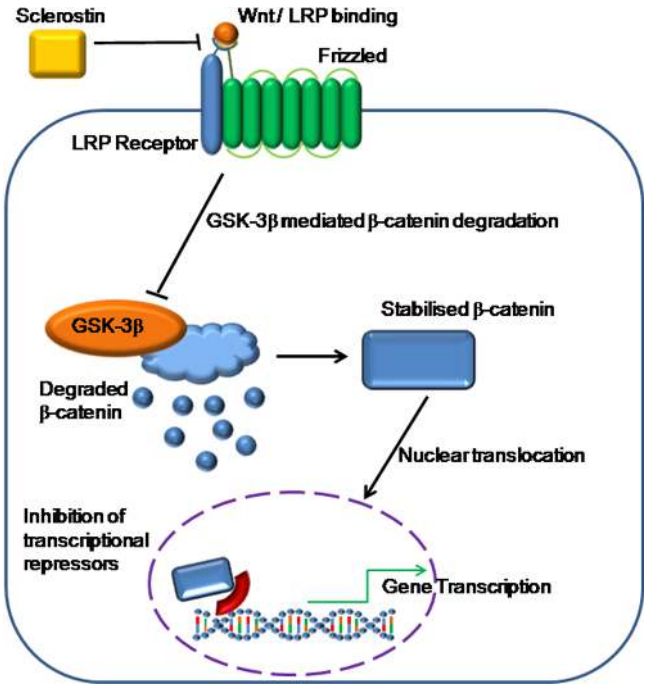



**Figure 12.3** Sclerostin inhibition of Wnt canonical pathway induction of osteogenesis. Sclerostin binding to LRP receptor prevents formation of a Wnt/FZD/LRP complex with GSK-3β resulting in degradation of β-catenin in the cytosol. Thus, transcriptional repressors of *Runx2* gene are not inhibited and *Runx2* gene transcription does not occur.

homeostasis toward bone resorption and the typical menopause-induced bone loss [60]. Equivalently in men undergoing androgen-deprivation therapy for prostate cancer, decreased testosterone levels lead to increased bone turnover and bone loss [61].

In addition to the interplay between osteoblasts and osteoclasts, osteocytes or terminally differentiated osteoblasts embedded within bone matrix are also involved in regulation of bone. Sclerostin encoded by the SOST gene and a potent inhibitor of bone formation is produced for the most part by embedded osteocytes and is secreted through the processes or dendrites of these cells [62]. It is also produced by other cells embedded in a matrix, such as cementocytes in teeth and hypertrophic chondrocytes during growth plate development and osteoarthritis [63,64]. Although expression of sclerostin mRNA has been reported in a number of other tissues post-natally, including heart, liver, and high levels in the kidneys [65], sclerostin protein has only been reported by cells within matrix-rich tissues such as bone [62]. Sclerostin inhibition of bone formation has previously been reported to function through inhibition of the Wnt canonical pathway [66].

## 12.2.3 Molecular regulation of osteogenic differentiation from mesenchymal stromal cells

The molecular cues required to direct MSCs to form osteoblasts are not fully understood. However, a number of factors necessary for the process have been identified. These include transcription factors such as Runt-related transcription factor 2 (RUNX2), growth factors such as BMPs and TGF-β, along with the Wnt pathway. Recently, the role of Wnt has received increased attention owing to the identification of novel therapeutic targets of the pathway, such as the generation of antibodies against the Wnt antagonists sclerostin and Dickkopf factor 1 (DKK1), which will be explored further later.

### 12.2.3.1 Wnt pathway

The Wnt canonical pathway is essential for lineage regulation of MSCs and osteochondral progenitors to an osteoblast phenotype (Figure 12.3) [67,68].

The pathway is evolutionarily highly conserved and plays an important role in cell-to-cell communication, cell proliferation, migration, and differentiation during development and healing [69,70]. Moreover, Wnts also prevent osteoblast and osteocyte apoptosis by both canonical-dependent and -independent mechanisms [71]. Secreted frizzled related protein 1 (SFRP-1), an antagonist of the Wnt canonical pathway, has also been suggested to play a role in the regulation of osteoblast/osteocyte apoptosis [72]. Understanding the role of inhibitors of the Wnt pathway, such as sclerostin, has been possible due to knockout models of the Wnt canonical pathway and has resulted in the development of a number of therapeutic avenues currently being explored in regenerative orthopedics [73–76].

A combination of canonical and noncanonical Wnt signaling is essential for normal cell physiology. The Wnt/β-catenin or canonical pathway is essential for bone development [77–79]. During activation of this pathway, Wnt binding to Frizzled (FZD) receptor and either of the low-density lipoprotein receptors (LRPs) 5 or 6 results in a number of intercellular events. These include stabilization and accumulation of β-catenin in the cytosol and its ultimate translocation to the nucleus [80]. Here, β-catenin displaces repressors of target genes normally involved in bone formation, including *RUNX2* and downstream genes of *RUNX2* such as *OC* [81–83].

A number of endogenous inhibitors of this pathway exist that primarily function by interfering with the formation of the Wnt/LRP/FZD complex [84]. These include sclerostin, DKK1 [85,86] and sclerostin-domain-containing protein-1 (SOST-dc1), also known as WISE [83]. The molecules act by binding to the LRP5 and LRP6 receptors, preventing Wnt binding to the LRP/FZD complex. Other inhibitors of this pathway include SFRP1 and Wnt inhibitory factor (WIF), which bind directly to Wnt [84,87–89], preventing its binding to receptors.

DKK1 and sclerostin antagonize the Wnt canonical pathway. The inhibitory function of SOST-dc1 has only been demonstrated in tooth development to date [90]. Although early invertebrates expressed DKK1 [91,92], sclerostin expression has only been reported in bony vertebrates, which has previously been suggested to be due to sclerostin function being specific to bone [93]. Interestingly, positive expression of sclerostin has also been detected from osteocytes of a *Tyrannosaurus rex* dinosaur, implying that dinosaur bone was regulated similarly to that of extant vertebrates, indicating the highly conserved nature of sclerostin [94].

Although DKK1, SOST-dc1, and sclerostin all affect the Wnt pathway in a similar way [95], the apparent specificity of sclerostin to bone seems to suggest a more targeted effect of sclerostin to bone. The role of the Wnt pathway is highlighted in studies of mesenchymal progenitor cell differentiation during vertebrate skeletogenesis. Inactivation of β-catenin results in chondrocyte differentiation instead of osteoblast differentiation in models of both endochondral and intramembranous ossification, indicating a role for β-catenin in the regulation of the switch between osteogenesis and chondrogenesis [67,96].

#### 12.2.3.2 TGF-β superfamily

The TGF-β superfamily of secreted molecules, including BMPs, has also been identified as a key factor in the induction of bone formation [97]. TGF-β/BMP signaling is involved in a wide array of cellular processes during bone formation, both prenatally and postnatally. TGF-β/BMPs act via two pathways during bone formation, which merge at the *RUNX*2 gene directing MSCs towards differentiation [97]. The first pathway is the canonical SMAD-dependent pathway in which isoforms of TGF-β and their receptors TGF-βRI (ALK5) and TGF-βRII (TGF-βR2) are essential for both intramembranous and endochondral ossification [98]. The noncanonical TGF-β pathway, which is not dependent on SMAD, is also essential for osteoblast differentiation [98]. The noncanonical pathways are activated by direct ligand-bound receptor activation to modulate downstream cellular processes [99]. *In vitro* analysis of murine osteoprogenitors deficient in TGF-βRI indicated that TGF-β is involved in proliferation, differentiation and osteoblast lineage commitment via selective MAPKs and SMADs 2/3 pathways [100]. The phenotype of animals with a TGF-βRI defect displayed skeletal abnormalities, including the presence of short and wide long bones and reduced bone collars and trabecular bone. Moreover, TGF-βRI-deficient mice possessed growth plates with unusually thin perichondral cell layers, which the authors suggested may have been associated with the short bones and ectopic cartilaginous protrusions observed [100]. This indicates the essential role of TGF-β signaling in perichondrium formation and differentiation and growth plate integrity during development [100].

BMPs belong to the TGF-β superfamily and are heavily involved in bone and cartilage formation. BMPs play a role in bone maintenance by binding to either BMP receptor type-1A or -1B (BMPR-1A or BMPR-1B) to direct osteoblast cell function [101]. BMPR-1A in mouse osteoblasts negatively regulated bone mass by upregulation of the WNT inhibitors sclerostin and DKK1 [102]. BMPs have also been shown to positively regulate expression of osteoblast proteins associated with bone formation such as ALP, OC, osterix, and RUNX2 [103,104]. Binding of BMPs to their receptors results in mobilization of receptor-regulated SMADs, namely SMADs 1, 2, 3, 5, and 8 [103]. These SMADs are phosphorylated and form a co-SMAD protein with SMAD 4 that is ultimately translocated to the nucleus, where it exerts its effect. At the nucleus, this co-SMAD protein interacts with the osteogenesis master gene *RUNX2* to facilitate gene transcription [103].

In addition to TGF-β signaling, a number of BMPs are involved in regulation of bone. Overexpression of BMP-6 and BMP-2 results in increased osteogenic differentiation *in vitro* and *in vivo*, with BMP-6 having the greatest effect [105]. BMP-2 is involved in initiating osteoblast differentiation and regulating bone mineral density [106]. Knockout studies indicate a role of BMP-2 and -4 in normal bone development and maintenance, since knockouts of these factors resulted in reduced osteogenesis and bone fracturing [98]. BMP-7, or osteogenic protein-1 (OP-1), has been used clinically for the treatment of long bone, scaphoid, and nonunion fractures. Similarly, there is supportive evidence for the use of BMP-7 as a therapy for spinal fusion and as a replacement for autografts [107]. However, BMP-7 knockdown does not result in an observable negative effect in bone development or maintenance [108]. Conversely, BMPs such as BMP-3 negatively regulate bone marrow stromal cells, with targeted deletion of BMP-3 resulting in increased trabecular bone loss and transgenic overexpression of BMP-3 leading to spontaneous fractures [109].

#### 12.2.3.3 Transcriptional regulators

The transcription factors that regulate osteoblast differentiation can be divided into two groups. The first are those that are specific to various stages of osteoblast differentiation and include RUNX2, osterix, and activating transcription factor 4. The second group of transcription factors are those that are not specific to osteoblasts but act downstream of other pathways but can nonetheless regulate the differentiation process. Examples of these include Twist-1 and Twist-2 [110,111].

*RUNX2*, also referred to as core-binding factor alpha 1, is widely considered the master gene for osteogenic differentiation, being essential for both intramembranous and endochondral bone formation [112]. *RUNX2* expression is typically observed with loss of expression of proliferator-activated receptor-γ (*PPAR-γ*), indicating a switch between differentiation of MSCs toward adipogenic or osteogenic lineages [113]. A number of downstream genes from *RUNX2*, including *OC*, *ALP*, bone sialoprotein (*BSP*), and osteopontin, are essential for various processes during bone formation, including osteoblast differentiation, production of matrix, and mineralization during bone formation. RUNX2 mediates its effect via a direct binding site called osteoblast-specific cis-acting element (OSE2) that is present in the osteoblast-specific genes mentioned earlier [114]. The essential role of RUNX2 has been evaluated in overexpression and knockdown studies. In CBAF1 null mice, neither endochondral nor intramembranous bone formation occurred due to an arrest in osteoblast differentiation [115,116]. Additionally, CBAF1-overexpressing chondrocytes underwent endochondral ossification at an accelerated rate [116]. These studies demonstrated the role of RUNX2 as a key regulator of both intramembranous and endochondral bone formation. Overexpression of RUNX2 in bone marrow-derived MSCs resulted in increased expression of ALP, OC, OP, and BSP [117]. Moreover, increased mineralization *in vitro* was also reported [117]. When MSCs overexpressing RUNX2 were used to treat a cranial defect in BALB/c mice, increased wound healing was observed [117]. These data indicate a role for RUNX2 in both *in vitro* and *in vivo* osteogenesis of MSCs.

As mentioned previously, Twist proteins have also been reported to regulate osteogenesis. Twist is a basic helix–loop–helix transcription factor that reduces mRNA levels of CBAF1 by binding to OSE2 in the OC reporter [118]. In *Drosophila*, Twist acts to regulate mesoderm formation and dorsal–ventral patterning. In mammals, Twist-1 and Twist-2 are essential for normal bone formation. In both human and mouse, Twist-1 heterozygotes exhibit premature osteoblast differentiation in the skull [119]. Additionally, Twist-1 and Twist-2 heterozygotes and Twist-2 homozygote negatives died early after birth due to activation of multiple cytokine pathways [119,120]. Twist suppresses osteogenesis through either RUNX2 or histone deacetylases, with a reduction in levels prior to the onset of osteogenesis [121].

The transcription factors discussed are involved in regulation of osteogenesis. However, additional transcription factors are involved in osteochondrogenesis and so also contribute to bone formation. These include SOX9 and musculoaponeurotic fibrosarcoma oncogene homologue [121,122].

### 12.2.4 Factors regulating homing of mesenchymal stromal cells to bone

MSCs exist within all vascularized tissues and organs and have been shown to be capable of homing to injured tissues. This has been observed in a number of injury models, including migration of MSCs to bone fractures [123–125], lung injury [126], brain injury [127], and to sites of wound healing [128]. Mobilization of MSCs to other injury sites has also been reported [129]. The mechanism of this homing of MSCs is the subject of an intense research effort in the hope of identifying a means of improving tissue repair by endogenous cells. The current consensus suggests that injured tissue produces factors and ligands that can be used by MSCs to migrate, adhere, and infiltrate damaged tissue [130–132], a process similar to that by leukocytes migrating to sites of inflammation. This idea was validated in a recent paper that described the migration of MSCs in an *in vitro* model [133]. MSC migration was dependent on their ability to adhere to, and migrate through, TNF-α-activated endothelium, a process dependent on both the G-protein-coupled receptor and vascular cell adhesion molecule (VCAM-1) pathways. The process is shown in Figure 12.4.

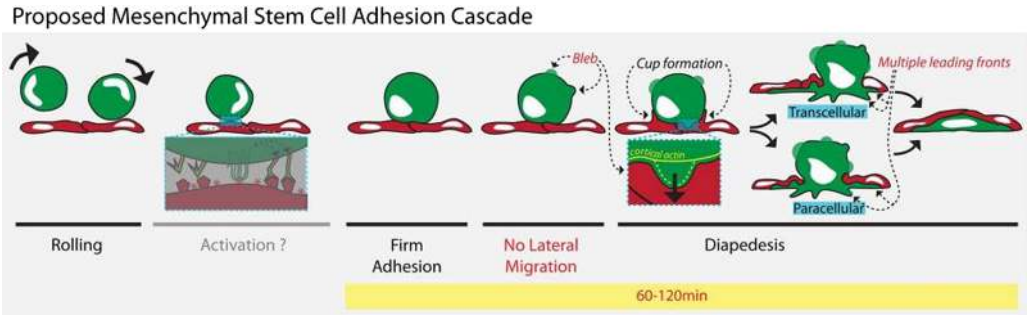



**Figure 12.4** Proposed mechanism of MSC migration to injured tissue in comparison with leukocyte migration to inflammation. (See color plate section.)
*Source*: Teo 2012 [133], pp. 2472–2486. Reproduced with permission of John Wiley and Sons.

First, rounded MSCs adhere to epithelium in an integrin-dependent manner. Next, a transmigratory cup is formed in which VCAM-1-enriched microvilli projections extend around the MSC and form a cup shape. The next step involves formation of a gap or pore by a process described as blebbing in which MSCs apply a force on the epithelium that is associated with the formation of gaps or pores. This stage is not observed in the migration of leukocytes, which migrate through intact gap junctions, a process known as diapedesis. Following this, MSCs penetrate and spread through the subendothelium layer and transmigration is complete [133].

Initiation of this migration is induced by chemokines and cytokines at the site of injury. A number of studies of the chemotactic effect of particular cytokines on the migration of MSCs have been performed. C–X–C chemokine receptor type 4 (CXCR4), the receptor for stromal-derived factor-1 (SDF-1) that is encoded by the C–X–C motif chemokine 12 (*CXCL12*) gene, has been associated with migration of murine and human MSCs from various sources, including bone marrow, adipose tissue, and umbilical cord [134–138]. In these studies, overexpression of CXCR4 enhanced the migratory ability of MSCs [139]. The migration of MSCs to sites of fracture healing is exclusively CXCR4 dependent, with migrated MSCs contributing to fracture repair by production of BMP-2 when they engraft in the fracture site [123]. Additionally, during healing of live bone grafts, increased expression of SDF-1 messenger RNA is observed in the periosteum, with the addition of anti-SDF-1 antibody inhibiting bone formation [140]. This study also reported the ability of CXCR4-expressing MSCs, which had homed to the site of bone injury, to contribute to bone repair via endochondral ossification [140]. In addition to CXCR4, a number of additional factors have been identified that can both enhance and reduce MSC migration [141]. In a study by Ozaki *et al* 26 growth factors were assessed for migration of human and rabbit MSCs. Factors that consistently enhanced MSC migration were platelet-derived growth factor (PDGF)-BB and -AB, epidermal growth factor, TGF-α, fibroblast growth factor-2, insulin-like growth factor, and hepatocyte growth factor, with PDGF-BB having the greatest effect on migration. Interestingly, combinations of growth factors that shared the same receptor did not result in an enhanced effect on migration of MSCs, while other combinations, such as FGF-2 and PDGF-BB, actually inhibited migration. This indicates a highly complex, regulated process of MSC migration (and see Chapters 22 and 23).

### 12.2.5 *In vivo* detection and contribution of mesenchymal stromal cells to osteogenesis

The potential of cell-based therapies for tissue regeneration is well accepted. Although the paradigm may have shifted from the initial belief that implanted cells fundamentally contribute to regeneration through direct incorporation into the tissue to a more complex understanding of the importance of indirect regeneration via paracrine mechanisms, the ability to monitor implanted cells in order to assess where they go and what they do when they arrive is necessary for the development of more successful therapies. A variety of modalities based on bioluminescent imaging, single-photon emission computed tomography, positron emission tomography, multiple photon microscopy, and magnetic resonance imaging are currently used for preclinical tracking of MSCs *in vivo*. Labeling of the cells is necessary for visualization and may include contrast reagents such as quantum dots [142,143], superparamagnetic iron oxide nanoparticles [144–146], or reporter genes such as luciferase and green fluorescent protein (GFP). Despite the apparent high level of choice of reagents, they are not without issues owing to the short half-life of the reagent [147], donor-to-host transfer [148–151], and virus toxicity [152].

While the clinical importance of *in vivo* tracking of MSCs is obvious, the contribution of the cells to tissue repair at their target destination requires clarification. Some common methods to address this include lineage mapping, sex-mismatched donor–recipient pairs, and detection of the human Alu sequence in human cells in the tissue of animal models [153–155]. In one study, human MSCs were transplanted subcutaneously into immune-compromised mice either undifferentiated or after *in vitro* chondrogenic preinduction for 6 weeks. Using *in situ* hybridization, the authors delineated the contribution of human cells to tissue formation via detection of human-specific Alu repeat sequences. Interestingly, the authors showed that after 8 weeks' subcutaneous implantation in mice, bone-lining cells producing new bone were of human origin while adjacent bone marrow was of murine origin [153]. Using a GFP reporter model, Kalajzic *et al.* identified a defined population of mesenchymal progenitor cells expressing α-smooth muscle actin (α-SMA) that were associated with the perivascular niche and demonstrated pro-osteogenic characteristics [156]. In a related study, Grcevic *et al.* created male α-smooth muscle actin promoter-directed Cre recombinase estrogen receptor ligand-binding domains (αSMACreERT2) transgenic mice that were crossed with female Ai9 reporter mice to allow temporal and spatial detection of the $\alpha SMA^+$ progenitor cells by tamoxifen-induced Cre recombinase activated tdTomato expression [155]. tdTomato is an exceptionally bright red fluorescent protein - 6 times brighter than EGFP. Using this approach the authors traced the $\alpha SMA^+$ progeny into mature osteoblasts and terminally differentiated osteocytes. Interestingly, in a tibial fracture repair model the majority of the callus cells, including chondrocytes and osteoblasts, were identified as $SMA9^+$-expressing cells [155]. Mizoguchi *et al.* used double-transgenic osx-creERT2/ROSA26-loxP-stop-loxP-tdTomato (iOsx/Tomato) reporter mice to trace osterix-expressing ($osx^+$) cells to delineate three distinct mesenchymal precursors in fetal, perinatal, and adult bone marrow [157]. To assess whether the iOsx/$Tomato^+$ stromal cells displayed mesenchymal stromal and progenitor cell characteristics, the authors then generated Nes-Gfp/iOsx/Tomato triple-transgenic mice. They observed that approximately 85% of iOsx/$Tomato^+$ bone marrow cells also expressed

Nes-GFP. In a previous study, the same group had identified leptin receptor positive cells ($Lepr^+$) overlapping with those cells of the bone marrow that were Nes-$GFP^+$ [158]. Interestingly, the majority of iOsx/$Tomato^+$ cells that were positive for Nes-GFP also expressed leptin receptor and previously identified mesenchymal stromal and progenitor cell markers [158]. However, Lepr expression was initially low in iOsx/$Tomato^+$ cells after 1 day of exposure to tamoxifen, which suggested a temporal increase in Lepr expression in the postnatal day 5 (P5)-iOsx/$Tomato^+$ bone marrow cells during bone maturation. The authors also investigated the contribution of $Lepr^+$ cells to bone tissues in Nes-Gfp/Lepr-cre/Tomato triple-transgenic mice and found that Lepr-cre-derived $Tomato^+$ cells (Lepr/$Tomato^+$) expressed Nes-GFP, PDGFR-α, and PDGFR-β, which have been previously identified as markers of mesenchymal stromal and progenitor cells [158]. Moreover, clonally derived Lepr/$Tomato^+$ cells were capable of trilineage differentiation, which the authors suggested implied that these populations associate with bone marrow P5-iOsx/$Tomato^+$ cells. Subsequent experiments showed that Lepr/$Tomato^+$ mature osteolineage cells were descendants of $Lepr^+$ precursors and that iOsx/$Tomato^+$ cells that co-expressed Lepr-cre appeared to be a source of osteoprogenitors that contributed to bone homeostasis. To further explore this relationship, a tibial fracture model was employed to determine the contribution of iOsx/$Tomato^+$ and $Lepr^+$ cells to bone regeneration. Both iOsx/$Tomato^+$ and lepr/$Tomato^+$ cells were identified within the periosteum, indicating a role in the fracture healing process. The iOsx/$Tomato^+$ cells were identified within the soft callus approximately 8 days after the fracture, and within this population a subset of cells also expressed SOX9. Lepr/$Tomato^+$ cells were also detected as $SOX9^+$ cells in the fracture callus in Lepr-cre/Tomato mice, thus supporting their role in bone regeneration [157] and correlating with findings of Ono *et al.* [154]. It will be of interest to assess the association between $iOsx^+$, $Lepr^+$, and $SMA9^+$ cell in a similar repair model.

### 12.2.6 Regulating the immune system for bone formation

In addition to their ability to differentiate into multiple tissue types, MSCs are also being considered as a therapeutic agent based on their immunomodulatory properties [159]. Osteoimmunology or how the immune system regulates bone formation is an emerging field in bone biology [160–163]. Numerous types of immune system cells, including B cells, T cells [164], natural killer cells [165], and dendritic cells [166], have all been reported to play a role in osteoclast function. The high level of interaction is not surprising as osteoclasts are terminally differentiated macrophages [166]. B cells have been reported to induce osteoclast apoptosis via production of TGF-β1 [165], reducing levels of bone resorption and hence increasing overall bone mass. The effect of cytokines secreted by cells of the immune system has also been reported to play a role on osteoblast function, although this has not been defined to the same extent as for osteoclasts. In addition to the inhibition of osteoblast differentiation by TNF-α [167], TNF-α, interferon-γ (IFN-γ), and IL-1 have all been reported to suppress collagen type I synthesis by osteoblasts [160,168–170]. There are conflicting data on the mechanism by which T cells activate osteoblastogenesis. However, it is logical to assume that any cell attempting to differentiate into bone in an environment at least partially governed by the immune system needs to be able to act in an immunomodulatory or suppressive manner. A growing body of *in vitro* and *in vivo* data now exists to support the idea that MSCs can act as immunosuppressive agents [159,171–174]. The low level of immunogenicity of MSCs as a consequence of their low expression of major histocompatibility complex (MHC) class I molecules and absence of MHC class II molecules and absence of CD40, CD80, and CD86 co-stimulatory molecules has been a driving force toward the clinical use of these cells [175–178]. Owing to this lack of immunogenicity, MSCs in theory should elicit little to no immune response by the recipient's immune system, which in turn suggests that allogeneic transplantation from unrelated and MHC-unmatched MSC donors should be clinically feasible. The mechanisms by which MSCs exert their immunomodulatory effects include both the production of soluble factors and direct cell-to-cell contact [179,180]; and see Chapters 33 and 34. There are a number of soluble factors produced by MSCs that play a role in MSC-mediated immunosuppression, including indoleamine 2,3-dioxygenase (IDO), TGF-β1, prostaglandin $E_2$, nitric oxide, hepatocyte growth factor, and human leukocyte antigen-G. The production of soluble immunosuppressive factors such as IDO requires initial activation of MSCs by immune-cell-mediated IFN-γ, which can work synergistically with a combination of IL-1α, IL-1β, and TNF-α [180,181]. T cells were the first cell of the immune system to be demonstrated to be suppressed by MSCs [182]. This effect has been observed with autologous, allogeneic, and xenogeneic MSCs [173,175]. Similarly, activated B cells are suppressed by MSCs in the presence of exogenous IFN-γ [181]. This effect is through release of factors that both increase B cell viability while also inhibiting proliferation and arresting the cells in the $G_0/G_1$ phase of the cell cycle [183].

## 12.3 Clinical applications of mesenchymal stromal cells in bone disorders

### 12.3.1 Bone regeneration

Clinical trials investigating the efficacy of MSCs in bone regeneration are limited to early phase I and II studies. Indications for these trials include avascular necrosis of bone (osteonecrosis) (NCT016105383), distraction osteogenesis with limb length discrepancies (NCT01210950), and fracture nonunion (NCT01206179, NCT01429012, NCT01842477). Completed trials investigating the use of autologous MSCs for the treatment of nonunion fractures have not yet been published (NCT01206179, NCT02177565). Quarto *et al.* have demonstrated healing of

nonunion fractures in a three-patient pilot study involving bone marrow-derived osteoprogenitors implanted in the defect site on a custom-fit hydroxyapatite scaffold [184]. Hernigou *et al.* treated 60 cases of tibial nonunion with a percutaneous infusion of concentrated autologous bone marrow cells [185]. Bone union was achieved in 53 patients. In those patients where bone union was not achieved, significantly fewer progenitor cells were present [185]. Similar successful outcomes using bone marrow-derived cells for the treatment of nonunion fractures have been reported with significant follow-up times [186]. Hibi *et al.* described the treatment of an alveolar cleft defect in a 9-year-old female patient with culture-expanded, osteogenically induced bone marrow-derived MSCs using platelet-rich plasma as a scaffold material [187]. Regenerated bone was evident 3 months after implantation with approximately 79% bridging observed after 6 months. Mesimäki *et al.* reported the first clinical case in which ectopic bone was produced using autologous adipose-derived stem cells (ADSCs) in a microvascular reconstruction study of the maxilla [188]. The expanded autologous ADSCs were seeded onto a β-tricalcium phosphate scaffold containing recombinant human BMP-2. This cell-seeded scaffold was implanted beneath a muscle flap of the rectus abdominus muscle, where it remained for 8 months. Integration was described as excellent at 4 months after implantation [188]. Determining the relative contribution of cells, scaffold, or growth factors to tissue regeneration in these studies is difficult. Several other small clinical studies using MSCs and ceramic scaffolds for the treatment of cranio-maxillofacial defects have been reported [189]. Generally, restorative dentistry has proved relatively successful for MSC-based bone tissue engineering approaches, most likely given the non-load-bearing nature of this anatomical site. The treatment of long bone defects, however, still remain largely centered on animal studies.

The lack of clinical activity in this specific area may arise from the challenges faced relating to treatment of bone defects in long bones, such as the size of the defect and the mechanical environment (i.e., high load). While several animal studies support the concept of a cell therapy approach for the treatment of bone defects [190,191], there are also other studies that failed to find a positive outcome of human bone marrow aspirate or MSCs in the treatment of femoral defects [192]. In recent years a more biologically tailored approach to MSC-based therapies for the treatment of long bone defects has been attempted. Specifically, chondrogenic predifferentiation of MSCs in an attempt to favor endochondral ossification within the defect site has been reported [153,191,193,194]. Van der Stok *et al.* implanted undifferentiated and *ex vivo* chondrogenically predifferentiated MSCs into a 6 mm rat femoral defect. In all cases chondrogenically differentiated pellets induced significantly more bone formation than undifferentiated MSCs over an 8-week period. The extent of bone formation, however, was observed to be donor dependent [193]. In a similar study, Harada *et al.* chondrogenically predifferentiated MSCs and then them seeded on to a polylactic-glycolic acid scaffold and implanted it into a 5 mm critical size defect and a 15 mm full-thickness defect in rats. At 4 weeks after implantation, immunohistological staining for collagen type II appeared to decline, with an associated increase in type I collagen staining. At 8 weeks after implantation the biomechanical strength of the 15 mm defect samples reached 75% that of the contralateral untreated femora and did not differ significantly from the 5 mm defect samples [194]. Janicki *et al.* found in a study which assessed heterotopic bone formation of chondrogenically predifferentiated human MSCs implanted subcutaneously in mice that the human MSCs contributed to bone formation observed, while the host cells contributed primarily to supporting tissues such as the marrow niche [153]. Tortelli *et al.* [191] described how commitment of implanted cells can influence the role of host versus implanted cells in bone formation as well as the type of ossification observed. Syngeneic (genetically identical) MSCs induced bone formation by endochondral ossification, while implanted osteoblasts led to intramembranous ossification. Notably, the bone formed in MSC-implanted mice appeared to be derived from host cells and was associated with increased recruitment of $CD31^+$ endothelial cells. In contrast, bone formed in the osteoblast-implanted group primarily displayed direct bone formation from the implanted donor cells without a noteworthy contribution of host cells [191].

### 12.3.2 Osteoarthritis

Osteoarthritis is a common but currently incurable disease. Treatment normally involves palliative measures to reduce pain and swelling, surgical intervention to increase longevity of the joint, and, finally, total joint replacement once joint destruction has occurred. Several clinical trials assessing the safety and efficacy of MSCs in the treatment of osteoarthritis have been completed or are under way. A major unexpected obstacle that emerged from some of the studies was failure to meet primary endpoints. Furthermore, the eventual fate and tissue destination of the injected cells has yet to be fully defined both in preclinical and clinical models [195,196]. There has more recently been a shift in focus to the immunomodulatory function of MSCs in tissue repair and how this may be best exploited therapeutically. This refocusing was brought about by increasing reports of reductions in inflammation, apoptosis, and fibrosis in the apparent absence of engraftment of MSCs in the target tissue. The trophic effects of MSCs, therefore, have become increasingly important for progressing the clinical application of MSCs. A recent paper describing injection of human MSCs in a hemimeniscectomy rat model demonstrated a cascade of gene expression associated with cartilage formation and repair [197]. It is likely that both the differentiation of MSCs into the desired cell type and their trophic effect on endogenous cells contribute to their therapeutic action, and it has been shown that these events are closely linked. For instance, recent studies have shown that trophic factors released by MSCs regulate the chondrocyte phenotype [198,199]. Moreover, the co-culture of human MSCs with osteoarthritis chondrocytes resulted in differentiation of MSCs toward chondrocytes. This occurred in the absence

of growth factors, and the co-culture condition prevented hypertrophic differentiation of the chondrocytes [200]. Another study also implied that this trophic effect extends to ADSCs in that the ADSCs decreased expression of hypertrophic and fibroblastic markers on osteoarthritis chondrocytes and the authors identified hepatocyte growth factor as a key mediator of this process [201].

### 12.3.3 Osteogenesis imperfecta

Osteogenesis imperfecta (OI) is a genetic disorder characterized by production of defective type I collagen, the principal structural protein of bone. OI patients are faced with frequent bone fractures, often without appreciable trauma, retarded bone growth, and progressive bone deformity. A characteristic clinical feature is blue, instead of white, sclerae of the eyes. The sclerae are thin and allow the color of the choroid membrane behind to be seen. No other disease has this feature (Figure 12.5).

Bisphosphonates have been found to be somewhat effective in the treatment of OI by strengthening the bones, reducing pain, and reducing the frequency of fracture. However, this disease remains incurable. Several early studies assessing the potential of stem cell therapy in OI focused on murine models [202]. Pereira *et al.* transplanted culture-expanded bone marrow-derived MSCs harvested from transgenic mice that expressed a type I collagen mini-gene into normal mouse recipients [203]. Up to 12% of donor cells were located in the connective tissues of recipient animals, suggesting that these cells can act as long-term precursors for connective tissues [203]. In another study, a significant, albeit small, increase in collagen and mineral content was reported 1 month after the MSCs were transplanted [204]. These findings formed the basis of a small clinical trial in three infants with severe OI [205]. At 3 months after allogenic hematopoietic stem cell transplantation, using bone marrow as the source, an increase in mineral density in all three patients was observed. In two of the three patients the growth velocity posttransplant was significantly increased and ultimately was found to be comparable to the normal median growth velocity of age- and sex-matched children. Furthermore, the frequency of fractures was significantly reduced in all three patients [205]. Interestingly, these substantial clinical outcomes were achieved with approximately 2% cell engraftment. In a follow-up study the authors assessed growth rate and mineral density in three severe OI infant patients (two from the original study) compared with two untreated age- and sex-matched patients at 18–36 months posttransplant of unmanipulated bone marrow. Similar to the pilot study, the authors observed increased rates of growth compared with controls and increased bone mineral density that reached the lower limit of the normal range. Again, similar to the pilot study, a decrease in fracture frequency in bone marrow-treated patients was observed [206]. Combined, these studies are indicative of a therapeutic effect of bone marrow-derived cells in reducing the severity of OI with increases in mineral content and growth and a reduced fracture frequency. The mechanism whereby this small population of engrafted cells contributed to the clinical effect observed remains to be elucidated.

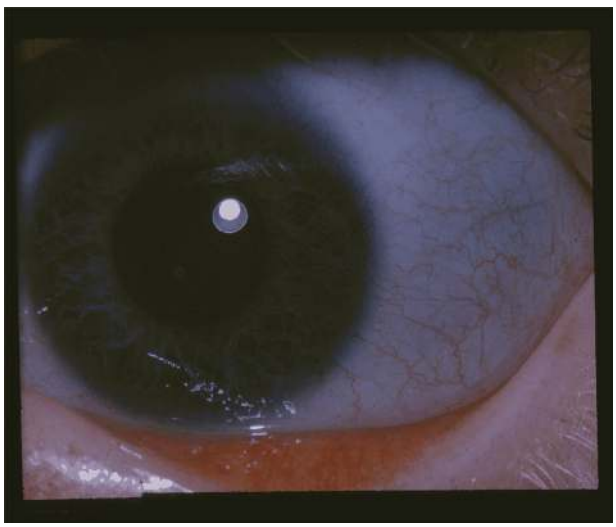


**Figure 12.5** The blue sclerae seen in OI. The patient was a 15-year-old male with OI who had been followed for some years because of fractures. The patient was first seen at age 30 months. He had a fracture of the right tibia at age 18 months without trauma. At 30 months he had a new fracture of the right tibia and a fracture of the left finger from a fall from a bicycle. The sclerae were noted to be blue. The blue coloration is caused by scleral thinness allowing the pigmented coat of the choroid to become visible. (See color plate section.) *Source*: "Osteogenesis sclerae" by National Eye Institute.

Le Blanc *et al.* successfully implanted *ex vivo* culture-expanded MSCs isolated from the liver of a 10-week-old aborted male fetus into a 32-week-old female fetus *in utero* that was diagnosed during gestation as having severe OI with multiple intrauterine fractures [207]. Nine months after birth a centromeric XY-specific probe on a bone biopsy sample revealed positive staining in approximately 0.3% of cells. The infant showed an increase in growth rate. The contribution of the engrafted cells, if any, was not defined [207].

The main question that arises from these studies is how so few engrafted cells achieved positive clinical outcomes. In all studies cited, analysis of cell engraftment took place at the earliest at 3 months after treatment. Initial cell engraftment may have been higher immediately postimplantation and may have declined with time [207,208]. A more recent line of thinking relates to the immunosuppressive function of MSCs and their ability to provoke an endogenous cell response [3,209]. The lack of categorical answers from clinical trial data has led researchers to reexamine animal models [208]. In a mouse model of OI, Niyibizi *et al.* tagged MSCs from normal mice with GFP which were then injected via the superficial temporal vein into immune-compromised mice. The vast majority of implanted cells reached the lungs and persisted there for up to 150 days [210]. However, 25 days after injection the donor cells were detected within the bone of several recipient mice. Donor cells were retrieved from these

mice, culture-expanded, and reinjected into new recipient mice. Interestingly, 35 days after reinjection the majority of donor cells were found in bone and were also found to migrate into cartilage, where they underwent chondrocyte differentiation [210]. Li *et al.* further identified subpopulations of MSCs that had a higher efficiency for migrating to bone and when injected colonized long bones of the mice where they differentiated into osteoblasts and deposited collagen type I, thus contributing to bone formation [211]. The authors noted that this subpopulation expressed RUNX2 and, therefore, may have represented an osteoprogenitor population rather than an undifferentiated MSC population. Otsuru *et al.* attempted to elucidate the mechanism by which whole bone marrow and *ex vivo* expanded MSCs exerted their clinical benefit [212]. They demonstrated in a mouse model of $CD3^+$ T cell-depleted nonadherent bone marrow cell transplantation that cells in the injected population were more potent osteoprogenitors than MSCs. They also observed that T cell-depleted bone marrow infusion in children with OI caused an increase in their rate of growth. Interestingly, in a murine model they suggested that the nonadherent bone marrow cells differentiated into osteoblasts and produced collagen type I, whereas MSCs secreted soluble factor(s) to indirectly stimulate growth [212].

## 12.4 Summary

The field of regenerative medicine has evolved exponentially over the last 20 years. Basic science has been accompanied by a multimillion-dollar business model that has led to ever-evolving stringent regulations. The commercialization of cell-based therapies has also been accompanied by a more sophisticated understanding of the cellular therapy paradigm. Where once it was thought that simple engraftment of these cells into an injury site would provide a reparative boost by direct transdifferentiation, more and more we are learning to understand the complexity of the bone marrow niche (see Chapter 32) and the importance of their trophic (see Chapters 18–21) and immunomodulatory effects (see Chapters 33 and 34). Nevertheless, in the context of bone regeneration, clinical application has been relatively slow. Interestingly, a severe genetic bone disease such as OI appears to be very responsive to cell-based therapies with minimal cell engraftment reported [206,207]. Recent studies assessing MSC populations in bone-related disease models have highlighted the importance of the MSC secretome (see Chapters 18 and 19) [213]. Moreover, lineage tracing studies have demonstrated the complexity of osteogenic differentiation of MSCs and the varying populations contributing to this [157,158]. Advances of cell-based therapies in bone regeneration have emerged with the realization that cell priming may provide a more robust endochondral bone formation response and that support of adequate angiogenesis is a key determinant in maintaining bone vitality [153,193]. Identification of pericytes and appreciation of the perivascular niche has been a significant development. Our once simple paradigm of cell engraftment for regeneration has been replaced by the need to understand and support a complex three-dimensional cell niche. Furthermore, the field has expanded to include the emergence of new and more sophisticated materials to help support osteogenic differentiation of MSCs. Given these developments, the onus on understanding where these cells go upon implantation and the biological role they play at their destination becomes fundamental to successful clinical application. This has led to the need for significant development in imaging and cell-tracking technologies. Regardless of the unresolved issues and need for constant adaptation and development to remain in sync with our increasing understanding of MSC biology, the studies that do demonstrate clinical efficacy suggest that it is just a matter of time before cell-based therapies for bone regeneration become a reality.

## References


1 Thomson JA, Itskovitz-Eldor J, Shapiro SS, *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* 1998;**282**(5391): 1145–1147.

2 Grayson WL, Bunnell BA, Martin E, *et al.* Stromal cells and stem cells in clinical bone regeneration. *Nat Rev Endocrinol* 2015;**11**: 140–150.

3 Barry F, Murphy M. Mesenchymal stem cells in joint disease and repair. *Nat Rev Rheumatol* 2013;**9**(10):584–594.

4 Yi T, Song SU. Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. *Arch Pharm Res* 2012;**35**(2):213–221.

5 De Peppo GM, Marolt D. Modulating the biochemical and biophysical culture environment to enhance osteogenic differentiation and maturation of human pluripotent stem cell-derived mesenchymal progenitors. *Stem Cell Res Ther* 2013;**4**(5): 106

6 Homma Y, Zimmermann G, Hernigou P. Cellular therapies for the treatment of non-union: the past, present and future. *Injury* 2013;**44**(Suppl 1):S46–S49.

7 Friedenstein AJ, Chailakhyan RK, Gerasimov UV. Bone marrow osteogenic stem cells: *in vitro* cultivation and transplantation in diffusion chambers. *Cell Prolif* 1987;**20**(3):263–272.

8 Johnstone B, Hering TM, Caplan AI, *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**(1):265–272.

9 Mackay AM, Beck SC, Murphy JM, *et al.* Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* 1998;**4**(4):415–428.

10 Gimble J, Guilak F. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 2003; **5**(5):362–369.

11 Gronthos S, Brahim J, Li W, *et al.* Stem cell properties of human dental pulp stem cells. *J Dent Res* 2002;**81**(8):531–535.

12 Fukuchi Y, Nakajima H, Sugiyama D, *et al.* Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 2004;**22**(5):649–658.

13 Mareschi K, Biasin E, Piacibello W, *et al.* Isolation of human mesenchymal stem cells: bone marrow versus umbilical cord blood. *Haematologica* 2001;**86**(10):1099–1100.

14 Zuk PA, Zhu M, Mizuno H, *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**(2):211–228.

15 Shi S, Gronthos S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003;**18**(4):696–704.

16 Mitsiadis TA, Barrandon O, Rochat A, *et al*. Stem cell niches in mammals. *Exp Cell Res* 2007;**313**(16):3377–3385.

17 Sacchetti B, Funari A, Michienzi S, *et al*. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**(2):324–336.

18 Da Silva Meirelles L, Caplan AI, Nardi NB. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 2008;**26**(9): 2287–2299.

19 Nakahara H, Goldberg VM, Caplan AI. Culture-expanded human periosteal-derived cells exhibit osteochondral potential *in vivo*. *J Orthop Res* 1991;**9**(4):465–476.

20 Yoshimura H, Muneta T, Nimura A, *et al*. Comparison of rat mesenchymal stem cells derived from bone marrow, synovium, periosteum, adipose tissue, and muscle. *Cell Tissue Res* 2007;**327**(3): 449–462.

21 Chang H, Knothe Tate ML. Concise review: the periosteum: tapping into a reservoir of clinically useful progenitor cells. *Stem Cells Transl Med* 2012;**1**(6):480–491.

22 Gerstenfeld LC, Cullinane DM, Barnes GL, *et al*. Fracture healing as a post-natal developmental process: molecular, spatial, and temporal aspects of its regulation. *J Cell Biochem* 2003;**88**(5):873–884.

23 Murao H, Yamamoto K, Matsuda S, Akiyama H. Periosteal cells are a major source of soft callus in bone fracture. *J Bone Miner Metab* 2013;**31**(4):390–398.

24 De Bari C, Dell'Accio F, Luyten FP. Human periosteum-derived cells maintain phenotypic stability and chondrogenic potential throughout expansion regardless of donor age. *Arthritis Rheum* 2001;**44**(1):85–95.

25 Sakaguchi Y, Sekiya I, Yagishita K, Muneta T. Comparison of human stem cells derived from various mesenchymal tissues: Superiority of synovium as a cell source. *Arthritis Rheum* 2005;**52**(8):2521–2529.

26 Bosch P, Musgrave DS, Lee JY, *et al*. Osteoprogenitor cells within skeletal muscle. *J Orthop Res*. 2000;**18**(6):933–944.

27 Jones EA, Kinsey SE, English A, *et al*. Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* 2002;**46**(12):3349–3360.

28 Cox G, Boxall SA, Giannoudis PV, *et al*. High abundance of $CD271^+$ multipotential stromal cells (MSCs) in intramedullary cavities of long bones. *Bone* 2012;**50**(2):510–517.

29 Tormin A, Li O, Brune JC, *et al*. CD146 expression on primary nonhematopoietic bone marrow stem cells is correlated with in situ localization. *Blood* 2011;**117**(19):5067–5077.

30 Okamoto K, Takayanagi H. Regulation of bone by the adaptive immune system in arthritis. *Arthritis Res Ther* 2011;**13**(3):219.

31 Weber DR, Econs MJ, Levine MA. Osteopetrosis: pathogenesis, management and future directions for research. *IBMS BoneKEy* 2014;**11**: article no. 520. doi: 10.1038/bonekey.2014.15

32 Caplan AI. Mesenchymal stem cells. *J Orthop Res* 1991;**9**(5): 641–650.

33 Väänänen HK, Laitala-Leinonen T. Osteoclast lineage and function. *Arch Biochem Biophys* 2008;**473**(2):132–138.

34 Matsuo K, Irie N. Osteoclast-osteoblast communication. *Arch Biochem Biophys* 2008;**473**(2):201–209.

35 Civitelli R, Beyer EC, Warlow PM, *et al*. Connexin43 mediates direct intercellular communication in human osteoblastic cell networks. *J Clin Invest* 1993;**91**(5):1888–1896.

36 Ilvesaro J, Väänänen K, Tuukkanen J. Bone-resorbing osteoclasts contain gap-junctional connexin-43. *J Bone Miner Res* 2000; **15**(5):919–926.

37 Kusu N, Laurikkala J, Imanishi M, *et al*. Sclerostin is a novel secreted osteoclast-derived bone morphogenetic protein antagonist with unique ligand specificity. *J Biol Chem* 2003;**278**(26): 24113–24117.

38 Boyce BF, Xing L. Biology of RANK, RANKL, and osteoprotegerin. *Arthritis Res Ther* 2007;**9**(Suppl 1):S1.

39 Kawai T, Matsuyama T, Hosokawa Y, *et al*. B and T lymphocytes are the primary sources of RANKL in the bone resorptive lesion of periodontal disease. *Am J Pathol* 2006;**169**(3):987–998.

40 Wright HL, McCarthy HS, Middleton J, Marshall MJ. RANK, RANKL and osteoprotegerin in bone biology and disease. *Curr Rev Musculoskelet Med* 2009;**2**(1):56–64.

41 Yasuda H, Shima N, Nakagawa N, *et al*. Identity of osteoclastogenesis inhibitory factor (OCIF) and osteoprotegerin (OPG): a mechanism by which OPG/OCIF inhibits osteoclastogenesis *in vitro*. *Endocrinology* 1998;**139**(3):1329–1337.

42 Min H, Morony S, Sarosi I, *et al*. Osteoprotegerin reverses osteoporosis by inhibiting endosteal osteoclasts and prevents vascular calcification by blocking a process resembling osteoclastogenesis. *J Exp Med* 2000;**192**(4):463–474.

43 Leibbrandt A, Penninger JM. RANK/RANKL: regulators of immune responses and bone physiology. *Ann N Y Acad Sci* 2008;**1143**:123–150.

44 Anderson DM, Maraskovsky E, Billingsley WL, *et al*. A homologue of the TNF receptor and its ligand enhance T-cell growth and dendritic-cell function. *Nature* 1997;**390**(6656):175–179.

45 Glass DA, Bialek P, Ahn JD, *et al*. Canonical Wnt signaling in differentiated osteoblasts controls osteoclast differentiation. *Dev Cell* 2005;**8**(5):751–764.

46 Raisz LG, Luben RA, Mundy GR, *et al*. Effect of osteoclast activating factor from human leukocytes on bone metabolism. *J Clin Invest* 1975;**56**(2):408–413.

47 Mori G, D'Amelio P, Faccio R, Brunetti G. The interplay between the bone and the immune system. *Clin Dev Immunol* 2013; **2013**:16.

48 Allen MJ, Myer BJ, Khokher AM, *et al*. Pro-inflammatory cytokines and the pathogenesis of Gaucher's disease: increased release of interleukin-6 and interleukin-10. *QJM* 1997;**90**(1):19–25.

49 Theill LE, Boyle WJ, Penninger JM. RANK-L and RANK: T cells, bone loss, and mammalian evolution. *Annu Rev Immunol* 2002; **20**(1):795–823.

50 Nakashima T, Takayanagi H. The dynamic interplay between osteoclasts and the immune system. *Arch Biochem Biophys* 2008;**473**(2):166–171.

51 Wuyts W, Van Wesenbeeck L, Morales-Piga A, *et al*. Evaluation of the role of RANK and OPG genes in Paget's disease of bone. *Bone* 2001;**28**(1):104–107.

52 Hughes AE, Ralston SH, Marken J, *et al*. Mutations in TNFRSF11A, affecting the signal peptide of RANK, cause familial expansile osteolysis. *Nat Genet* 2000;**24**(1):45–48.

53 Sobacchi C, Frattini A, Orchard P, *et al.* The mutational spectrum of human malignant autosomal recessive osteopetrosis. *Hum Mol Genet* 2001;**10**(17):1767–1773.

54 Bendre MS, Montague DC, Peery T, *et al.* Interleukin-8 stimulation of osteoclastogenesis and bone resorption is a mechanism for the increased osteolysis of metastatic bone disease. *Bone* 2003;**33** (1):28–37.

55 Huang JC, Sakata T, Pfleger LL, *et al.* PTH differentially regulates expression of RANKL and OPG. *J Bone Miner Res* 2004;**19** (2):235–244.

56 Koenders MI, Lubberts E, Oppers-Walgreen B, *et al.* Blocking of interleukin-17 during reactivation of experimental arthritis prevents joint inflammation and bone erosion by decreasing RANKL and interleukin-1. *Am J Pathol* 2005;**167**(1):141–149.

57 Wada T, Nakashima T, Hiroshi N, Penninger JM. RANKL–RANK signaling in osteoclastogenesis and bone disease. *Trends Mol Med* 2006;**12**(1):17–25.

58 Looker AC, Orwoll ES, Johnston CC, Jr. *et al.* Prevalence of low femoral bone density in older U.S. adults from NHANES III. *J Bone Miner Res* 1997;**12**(11):1761–1768.

59 Riggs BL, Khosla S, Melton LJ. A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II Osteoporosis in postmenopausal women and contributes to bone loss in aging men. *J Bone Miner Res* 1998;**13**(5):763–773.

60 Mazziotti G, Bilezikian J, Canalis E, *et al.* New understanding and treatments for osteoporosis. *Endocrine* 2012;**41**(1):58–69.

61 Mazziotti G, Canalis E, Giustina A. Drug-induced osteoporosis: mechanisms and clinical implications. *Am J Med* 2010;**123** (10):877–884.

62 Poole KES, van Bezooijen RL, Loveridge N, *et al.* Sclerostin is a delayed secreted product of osteocytes that inhibits bone formation. *FASEB J* 2005;**19**(13):1842–1844.

63 Chan BY, Fuller ES, Russell AK, *et al.* Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. *Osteoarthritis Cartilage* 2011;**19**(7):874–885.

64 Van Bezooijen RL, Bronckers AL, Gortzak RA, *et al.* Sclerostin in mineralized matrices and van Buchem disease. *J Dent Res.* 2009; **88**(6):569–574.

65 Moester MJ, Papapoulos SE, Lowik CW, van Bezooijen RL. Sclerostin: current knowledge and future perspectives. *Calcif Tissue Int* 2010;**87**(2):99–107.

66 Li X, Zhang Y, Kang H, *et al.* Sclerostin binds to LRP5/6 and antagonizes canonical Wnt signaling. *J Biol Chem* 2005; **280**(20):19883–19887.

67 Hill TP, Später D, Taketo MM, *et al.* Canonical Wnt/β-catenin signaling prevents osteoblasts from differentiating into chondrocytes. *Dev Cell* 2005;**8**(5):727–738.

68 Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* 2005; **8**(5):739–750.

69 Logan CY, Nusse R. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 2004;**20**:781–810.

70 Komiya Y, Habas R. Wnt signal transduction pathways. *Organogenesis* 2008;**4**(2):68–75.

71 Almeida M, Han L, Bellido T, *et al.* Wnt proteins prevent apoptosis of both uncommitted osteoblast progenitors and differentiated osteoblasts by β-catenin-dependent and -independent signaling cascades involving Src/ERK and phosphatidylinositol 3-kinase/AKT. *J Biol Chem* 2005;**280**(50):41342–41351.

72 Bodine PVN, Billiard J, Moran RA, *et al.* The Wnt antagonist secreted frizzled-related protein-1 controls osteoblast and osteocyte apoptosis. *J Cell Biochem* 2005;**96**(6):1212–1230.

73 Loots GG, Kneissel M, Keller H, *et al.* Genomic deletion of a long-range bone enhancer misregulates sclerostin in Van Buchem disease. *Genome Res* 2005;**15**(7):928–935.

74 Li X, Ominsky MS, Niu Q-T, *et al.* Targeted deletion of the sclerostin gene in mice results in increased bone formation and bone strength. *J Bone Miner Res* 2008;**23**(6):860–869.

75 Holmen SL, Giambernardi TA, Zylstra CR, *et al.* Decreased BMD and limb deformities in mice carrying mutations in both Lrp5 and Lrp6. *J Bone Miner Res* 2004;**19**(12):2033–2040.

76 Joeng KS, Schumacher CA, Zylstra-Diegel CR, *et al.* Lrp5 and Lrp6 redundantly control skeletal development in the mouse embryo. *Dev Biol* 2011;**359**(2):222–229.

77 Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, *et al.* Wnt/β-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* 2006;**281**(42):31720–31728.

78 Holmen SL, Zylstra CR, Mukherjee A, *et al.* Essential role of β-catenin in postnatal bone acquisition. *J Biol Chem* 2005;**280** (22):21162–21168.

79 Johnson ML, Harnish K, Nusse R, van Hul W. LRP5 and Wnt signaling: a union made for bone. *J Bone Miner Res* 2004;**19** (11):1749–1757.

80 Krishnan V, Bryant HU, Macdougald OA. Regulation of bone mass by Wnt signaling. *J Clin Invest* 2006;**116**(5):1202–1209.

81 Krause C, Korchynskyi O, de Rooij K, *et al.* Distinct modes of inhibition by sclerostin on bone morphogenetic protein and Wnt signaling pathways. *J Biol Chem* 2010;**285**(53):41614–41626.

82 Gaur T, Lengner CJ, Hovhannisyan H, *et al.* Canonical WNT signaling promotes osteogenesis by directly stimulating Runx2 gene expression. *J Biol Chem* 2005;**280**(39):33132–33140.

83 Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ. Update on Wnt signaling in bone cell biology and bone disease. *Gene* 2012;**492**(1):1–18.

84 Kular J, Tickner J, Chim SM, Xu J. An overview of the regulation of bone remodelling at the cellular level. *Clin Biochem* 2012;**45** (12):863–873.

85 Qiang YW, Barlogie B, Rudikoff S, Shaughnessy JD. Dkk1-induced inhibition of Wnt signaling in osteoblast differentiation is an underlying mechanism of bone loss in multiple myeloma. *Bone* 2008;**42**(4):669–680.

86 Guo J, Liu M, Yang D, *et al.* Suppression of Wnt signaling by Dkk1 attenuates PTH-mediated stromal cell response and new bone formation. *Cell Metab* 2010;**11**(2):161–171.

87 Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. *Gene* 2004;**341**:19–39.

88 Kubota T, Michigami T, Ozono K. Wnt signaling in bone metabolism. *J Bone Miner Metab* 2009;**27**(3):265–271.

89 Malinauskas T, Aricescu AR, Lu W, *et al.* Modular mechanism of Wnt signaling inhibition by Wnt inhibitory factor 1. *Nat Struct Mol Biol* 2011;**18**(8):886–893.

90 Ahn Y, Sanderson BW, Klein OD, Krumlauf R. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* 2010; **137**(19):3221–3231.

91 Augustin R, Franke A, Khalturin K, *et al*. Dickkopf related genes are components of the positional value gradient in *Hydra*. *Dev Biol* 2006;**296**(1):62–70.

92 Guder C, Pinho S, Nacak TG, *et al*. An ancient Wnt–Dickkopf antagonism in *Hydra*. *Development* 2006;**133**(5):901–911.

93 Ke HZ, Richards WG, Li X, Ominsky MS. Sclerostin and Dickkopf-1 as therapeutic targets in bone diseases. *Endocr Rev* 2012;**33**(5):747–783.

94 Schweitzer MH, Zheng W, Cleland TP, Bern M. Molecular analyses of dinosaur osteocytes support the presence of endogenous molecules. *Bone* 2013;**52**(1):414–423.

95 Baron R, Rawadi G. Targeting the Wnt/β-catenin pathway to regulate bone formation in the adult skeleton. *Endocrinology* 2007;**148**(6):2635–2643.

96 Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell* 2005;**8**(5):739–750.

97 Wu X, Shi W, Cao X. Multiplicity of BMP signaling in skeletal development. *Ann N Y Acad Sci* 2007;**1116**:29–49.

98 Chen G, Deng C, Li YP. TGF-β and BMP signaling in osteoblast differentiation and bone formation. *Int J Biol Sci* 2012;**8**(2):272–288.

99 Zhang YE. Non-Smad pathways in TGF-β signaling. *Cell Res* 2009;**19**(1):128–139.

100 Matsunobu T, Torigoe K, Ishikawa M, *et al*. Critical roles of the TGF-β type I receptor ALK5 in perichondrial formation and function, cartilage integrity, and osteoblast differentiation during growth plate development. *Dev Biol* 2009;**332**(2):325–338.

101 Lowery JW, Pazin D, Intini G, *et al*. The role of BMP2 signaling in the skeleton. *Crit Rev Eukaryot Gene Expr* 2011;**21**(2):177–185.

102 Kamiya N, Kobayashi T, Mochida Y, *et al*. Wnt inhibitors Dkk1 and Sost are downstream targets of BMP signaling through the type IA receptor (BMPRIA) in osteoblasts. *J Bone Miner Res* 2010;**25**(2):200–210.

103 Proff P, Romer P. The molecular mechanism behind bone remodelling: a review. *Clin Oral Investig* 2009;**13**(4):355–362.

104 Szczesny G. Molecular aspects of bone healing and remodeling. *Pol J Pathol* 2002;**53**(3):145–153.

105 Mizrahi O, Sheyn D, Tawackoli W, *et al*. BMP-6 is more efficient in bone formation than BMP-2 when overexpressed in mesenchymal stem cells. *Gene Ther* 2013;**20**(4):370–307

106 Bragdon B, Bonor J, Shultz KL, *et al*. Bone morphogenetic protein receptor type Ia localization causes increased BMP2 signaling in mice exhibiting increased peak bone mass phenotype. *J Cell Physiol* 2012;**227**(7):2870–2879.

107 White AP, Vaccaro AR, Hall JA, *et al*. Clinical applications of BMP-7/OP-1 in fractures, nonunions and spinal fusion. *Int Orthop* 2007;**31**(6):735–741.

108 Tsuji K, Cox K, Gamer L, *et al*. Conditional deletion of BMP7 from the limb skeleton does not affect bone formation or fracture repair. *J Orthop Res* 2010;**28**(3):384–389.

109 Kokabu S, Gamer L, Cox K, *et al*. BMP3 suppresses osteoblast differentiation of bone marrow stromal cells via interaction with Acvr2b. *Mol Endocrinol* 2012;**26**(1):87–94.

110 El Ghouzzi V, Le Merrer M, Perrin-Schmitt F, *et al*. Mutations of the *TWIST* gene in the Saethre–Chotzen syndrome. *Nat Genet* 1997;**15**(1):42–46.

111 Karsenty G, Kronenberg HM, Settembre C. Genetic control of bone formation. *Annu Rev Cell Dev Biol* 2009;**25**:629–648.

112 Lee KS, Kim HJ, Li QL, *et al*. Runx2 is a common target of transforming growth factor β1 and bone morphogenetic protein 2, and cooperation between Runx2 and Smad5 induces osteoblast-specific gene expression in the pluripotent mesenchymal precursor cell line C2c12. *Mol Cell Biol* 2000;**20**(23):8783–8792.

113 Li X, Cui Q, Kao C, *et al*. Lovastatin inhibits adipogenic and stimulates osteogenic differentiation by suppressing PPARγ2 and increasing Cbfa1/Runx2 expression in bone marrow mesenchymal cell cultures. *Bone* 2003;**33**(4):652–659.

114 Liu TM, Lee EH. Transcriptional regulatory cascades in Runx2-dependent bone development. *Tissue Eng Part B Rev* 2013;**19**(3):254–263.

115 Otto F, Thornell AP, Crompton T, *et al*. *Cbfa1*, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. *Cell* 1997;**89**(5):765–771.

116 Takeda S, Bonnamy JP, Owen MJ, *et al*. Continuous expression of *Cbfa1* in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev* 2001;**15**(4):467–481.

117 Zheng H, Guo Z, Ma Q, *et al*. Cbfa1/osf2 transduced bone marrow stromal cells facilitate bone formation *in vitro* and *in vivo*. *Calcif Tissue Int* 2004;**74**(2):194–203.

118 Yousfi M, Lasmoles F, Marie PJ. TWIST inactivation reduces CBFA1/RUNX2 expression and DNA binding to the osteocalcin promoter in osteoblasts. *Biochem Biophys Res Commun* 2002;**297**(3):641–644.

119 Kronenberg HM. *Twist* genes regulate Runx2 and bone formation. *Dev Cell* 2004;**6**(3):317–318.

120 Šošić D, Richardson JA, Yu K, *et al*. Twist regulates cytokine gene expression through a negative feedback loop that represses NF-κB activity. *Cell* 2003;**112**(2):169–180.

121 Frith J, Genever P. Transcriptional control of mesenchymal stem cell differentiation. *Transfus Med Hemother* 2008;**35**(3):216–227.

122 Hidaka C, Goldring MB. Regulatory mechanisms of chondrogenesis and implications for understanding articular cartilage homeostasis. *Curr Rheumatol Rev* 2008;**4**(3):136–147.

123 Granero-Molto F, Weis JA, Miga MI, *et al*. Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells* 2009;**27**(8):1887–1898.

124 Lee SW, Padmanabhan P, Ray P, *et al*. Stem cell-mediated accelerated bone healing observed with *in vivo* molecular and small animal imaging technologies in a model of skeletal injury. *J Orthop Res* 2009;**27**(3):295–302.

125 Kumagai K, Vasanji A, Drazba JA, *et al*. Circulating cells with osteogenic potential are physiologically mobilized into the fracture healing site in the parabiotic mice model. *J Orthop Res* 2008;**26**(2):165–175.

126 Ortiz LA, Gambelli F, McBride C, *et al*. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**(14):8407–8411.

127 Ji JF, He BP, Dheen ST, Tay SS. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* 2004;**22**(3):415–427.

128 Fu X, Han B, Cai S, *et al*. Migration of bone marrow-derived mesenchymal stem cells induced by tumor necrosis factor-α and its possible role in wound healing. *Wound Repair Regen* 2009;**17** (2):185–191.

129 Fong EL, Chan CK, Goodman SB. Stem cell homing in musculoskeletal injury. *Biomaterials* 2011;**32**(2):395–409.

130 Abbott JD, Huang Y, Liu D, *et al*. Stromal cell-derived factor-1α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation* 2004;**110**(21):3300–3305.

131 Wang Y, Deng Y, Zhou G-Q. SDF-1α/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. *Brain Res* 2008;**1195**: 104–112.

132 Schenk S, Mal N, Finan A, *et al*. Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* 2007;**25**(1):245–251.

133 Teo GS, Ankrum JA, Martinelli R, *et al*. Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells* 2012;**30**(11):2472–2486.

134 Park SA, Ryu CH, Kim SM, *et al*. CXCR4-transfected human umbilical cord blood-derived mesenchymal stem cells exhibit enhanced migratory capacity toward gliomas. *Int J Oncol* 2011;**38**(1):97–103.

135 Yu X, Chen D, Zhang Y, *et al*. Overexpression of CXCR4 in mesenchymal stem cells promotes migration, neuroprotection and angiogenesis in a rat model of stroke. *J Neurol Sci* 2012;**316** (1–2):141–149.

136 Liu X, Duan B, Cheng Z, *et al*. SDF-1/CXCR4 axis modulates bone marrow mesenchymal stem cell apoptosis, migration and cytokine secretion. *Protein Cell* 2011;**2**(10):845–854.

137 Jones GN, Moschidou D, Lay K, *et al*. Upregulating CXCR4 in human fetal mesenchymal stem cells enhances engraftment and bone mechanics in a mouse model of osteogenesis imperfecta. *Stem Cells Transl Med* 2012;**1**(1):70–78.

138 Bobis-Wozowicz S, Miekus K, Wybieralska E, *et al*. Genetically modified adipose tissue-derived mesenchymal stem cells overexpressing CXCR4 display increased motility, invasiveness, and homing to bone marrow of NOD/SCID mice. *Exp Hematol* 2011;**39**(6): 686.e4-696.e4.

139 Cheng Z, Ou L, Zhou X, *et al*. Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Mol Ther* 2008;**16**(3): 571–579.

140 Kitaori T, Ito H, Schwarz EM, *et al*. Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum* 2009;**60**(3):813–23.

141 Ozaki Y, Nishimura M, Sekiya K, *et al*. Comprehensive analysis of chemotactic factors for bone marrow mesenchymal stem cells. *Stem Cells Dev* 2007;**16**(1):119–129.

142 Muller-Borer BJ, Collins MC, Gunst PR, Cascio WE, Kypson AP. Quantum dot labeling of mesenchymal stem cells. *J Nanobiotechnol* 2007;**5**:9.

143 Jaiswal JK, Mattoussi H, Mauro JM, Simon SM. Long-term multiple color imaging of live cells using quantum dot bioconjugates. *Nat Biotechnol* 2003;**21**(1):47–51.

144 Van Buul GM, Kotek G, Wielopolski PA, *et al*. Clinically translatable cell tracking and quantification by MRI in cartilage repair using superparamagnetic iron oxides. *PLoS ONE* 2011;**6**(2):e17001.

145 Balakumaran A, Pawelczyk E, Ren J, *et al*. Superparamagnetic iron oxide nanoparticles labeling of bone marrow stromal (mesenchymal) cells does not affect their "stemness". *PLoS ONE* 2010;**5**(7): e11462.

146 Fan J, Tan Y, Jie L, *et al*. Biological activity and magnetic resonance imaging of superparamagnetic iron oxide nanoparticles-labeled adipose-derived stem cells. *Stem Cell Res Ther* 2013;**4**(2):44.

147 Lin CS, Xin ZC, Dai J, Lue TF. Commonly used mesenchymal stem cell markers and tracking labels: limitations and challenges. *Histol Histopathol* 2013;**28**(9):1109–1116.

148 Schormann W, Hammersen FJ, Brulport M, *et al*. Tracking of human cells in mice. *Histochem Cell Biol* 2008;**130**(2):329–338.

149 Li N, Yang H, Lu L, *et al*. Comparison of the labeling efficiency of BrdU, DiI and FISH labeling techniques in bone marrow stromal cells. *Brain Res* 2008;**1215**:11–19.

150 Iwashita Y, Crang AJ, Blakemore WF. Redistribution of bisbenzimide Hoechst 33342 from transplanted cells to host cells. *Neurreport*. 2000;**11**(5):1013–1016.

151 Castanheira P, Torquetti LT, Magalhas DR, *et al*. DAPI diffusion after intravitreal injection of mesenchymal stem cells in the injured retina of rats. *Cell Transplant* 2009;**18**(4):423–431.

152 Ji F, Duan HG, Zheng CQ, Li J. Comparison of chloromethyldialkylcarbocyanine and green fluorescent protein for labeling human umbilical mesenchymal stem cells. *Biotechnol Lett* 2015;**37**(2):437–447.

153 Janicki P, Kasten P, Kleinschmidt K, *et al*. Chondrogenic preinduction of human mesenchymal stem cells on β-TCP: enhanced bone quality by endochondral heterotopic bone formation. *Acta Biomater* 2010;**6**(8):3292–3301.

154 Ono N, Ono W, Nagasawa T, Kronenberg HM. A subset of chondrogenic cells provides early mesenchymal progenitors in growing bones. *Nat Cell Biol* 2014;**16**(12):1157–1167.

155 Grcevic D, Pejda S, Matthews BG, *et al*. *In vivo* fate mapping identifies mesenchymal progenitor cells. *Stem Cells* 2012;**30** (2):187–196.

156 Kalajzic Z, Li H, Wang LP, *et al*. Use of an alpha-smooth muscle actin GFP reporter to identify an osteoprogenitor population. *Bone* 2008;**43**(3):501–510.

157 Mizoguchi T, Pinho S, Ahmed J, *et al*. Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev Cell* 2014;**29**(3):340–349.

158 Pinho S, Lacombe J, Hanoun M, *et al*. PDGFRα and CD51 mark human nestin$^{+}$ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med* 2013;**210** (7):1351–1367.

159 Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007;**110**(10):3499–3506.

160 Walsh MC, Kim N, Kadono Y, *et al*. Osteoimmunology: interplay between the immune system and bone metabolism. *Annu Rev Immunol* 2006;**24**:33–63.

161 Takayanagi H. Interaction between the immune system and bone metabolism: an emerging field of osteoimmunology. *Proc Jpn Acad Ser B Phys Biol Sci* 2007;**83**(5):136–143.

162 Takayanagi H. Inflammatory bone destruction and osteoimmunology. *J Periodontal Res* 2005;**40**(4):287–293.

163 Caetano-Lopes J, Canhao H, Fonseca JE. Osteoimmunology – the hidden immune regulation of bone. *Autoimmun Rev* 2009;**8**(3):250–255.

164 Kotake S, Udagawa N, Hakoda M, *et al*. Activated human T cells directly induce osteoclastogenesis from human monocytes: possible role of T cells in bone destruction in rheumatoid arthritis patients. *Arthritis Rheum* 2001;**44**(5):1003–1012.

165 Weitzmann MN, Cenci S, Haug J, *et al*. B lymphocytes inhibit human osteoclastogenesis by secretion of TGFβ. *J Cell Biochem* 2000;**78**(2):318–324.

166 Wythe SE, Nicolaidou V, Horwood NJ. Cells of the immune system orchestrate changes in bone cell function. *Calcif Tissue Int* 2014;**94**(1):98–111.

167 Gilbert L, He X, Farmer P, *et al*. Inhibition of osteoblast differentiation by tumor necrosis factor-α. *Endocrinology* 2000;**141**(11):3956–3964.

168 Canalis E. Interleukin-1 has independent effects on deoxyribonucleic acid and collagen synthesis in cultures of rat calvariae. *Endocrinology* 1986;**118**(1):74–81.

169 Smith DD, Gowen M, Mundy GR. Effects of interferon-γ and other cytokines on collagen synthesis in fetal rat bone cultures. *Endocrinology* 1987;**120**(6):2494–2499.

170 Centrella M, McCarthy TL, Canalis E. Tumor necrosis factor-α inhibits collagen synthesis and alkaline phosphatase activity independently of its effect on deoxyribonucleic acid synthesis in osteoblast-enriched bone cell cultures. *Endocrinology* 1988;**123**(3):1442–1448.

171 Yañez R, Lamana ML, García-Castro J, *et al*. Adipose tissue-derived mesenchymal stem cells have *in vivo* immunosuppressive properties applicable for the control of the graft-versus-host disease. *Stem Cells* 2006;**24**(11):2582–2591.

172 Abumaree M, Al Jumah M, Pace R, Kalionis B. Immunosuppressive properties of mesenchymal stem cells. *Stem Cell Rev Rep* 2012;**8**(2):375–392.

173 Djouad F, Plence P, Bony C, *et al*. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;**102**(10):3837–3844.

174 Sudres M, Norol F, Trenado A, *et al*. Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. *J Immunol* 2006;**176**(12):7761–7767.

175 Pontikoglou C, Deschaseaux F, Sensebe L, Papadaki HA. Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. *Stem Cell Rev* 2011;**7**(3):569–589.

176 Uccelli A, Moretta L, Pistoia V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;**8**(9):726–736.

177 Weil BR, Manukyan MC, Herrmann JL, *et al*. The immunomodulatory properties of mesenchymal stem cells: implications for surgical disease. *J Surg Res* 2011;**167**(1):78–86.

178 Shi M, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic application of mesenchymal stem cells. *Clin Exp Immunol* 2011;**164**(1):1–8.

179 Ma S, Xie N, Li W, *et al*. Immunobiology of mesenchymal stem cells. *Cell Death Differ* 2014;**21**(2):216–225.

180 Ren G, Zhang L, Zhao X, *et al*. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**(2):141–150.

181 Krampera M, Cosmi L, Angeli R, *et al*. Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**(2):386–398.

182 Tse WT, Pendleton JD, Beyer WM, *et al*. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003;**75**(3):389–397.

183 Tabera S, Perez-Simon JA, Diez-Campelo M, *et al*. The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. *Haematologica* 2008;**93**(9):1301–1309.

184 Quarto R, Mastrogiacomo M, Cancedda R, *et al*. Repair of large bone defects with the use of autologous bone marrow stromal cells. *N Engl J Med* 2001;**344**(5):385–386.

185 Hernigou P, Poignard A, Beaujean F, Rouard H. Percutaneous autologous bone-marrow grafting for nonunions. Influence of the number and concentration of progenitor cells. *J Bone Joint Surg Am* 2005;**87**(7):1430–1437.

186 Marcacci M, Kon E, Moukhachev V, *et al*. Stem cells associated with macroporous bioceramics for long bone repair: 6- to 7-year outcome of a pilot clinical study. *Tissue Eng* 2007;**13**(5):947–955.

187 Hibi H, Yamada Y, Ueda M, Endo Y. Alveolar cleft osteoplasty using tissue-engineered osteogenic material. *Int J Oral Maxillofac Surg* 2006;**35**(6):551–555.

188 Mesimäki K, Lindroos B, Tornwall J, *et al*. Novel maxillary reconstruction with ectopic bone formation by GMP adipose stem cells. *Int J Oral Maxillofac Surg* 2009;**38**(3):201–209.

189 Chatterjea A, Meijer G, van Blitterswijk C, de Boer J. Clinical application of human mesenchymal stromal cells for bone tissue engineering. *Stem Cells Int* 2010;**2010**:215625.

190 Bruder SP, Kraus KH, Goldberg VM, Kadiyala S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* 1998;**80**(7):985–996.

191 Tortelli F, Tasso R, Loiacono F, Cancedda R. The development of tissue-engineered bone of different origin through endochondral and intramembranous ossification following the implantation of mesenchymal stem cells and osteoblasts in a murine model. *Biomaterials* 2010;**31**(2):242–249.

192 Cuomo AV, Virk M, Petrigliano F, *et al*. Mesenchymal stem cell concentration and bone repair: potential pitfalls from bench to bedside. *J Bone Joint Surg Am* 2009;**91**(5):1073–1083.

193 Van der Stok J, Koolen MK, Jahr H, *et al*. Chondrogenically differentiated mesenchymal stromal cell pellets stimulate endochondral bone regeneration in critical-sized bone defects. *Eur Cells Mater* 2014;**27**:137–148, discussion 148.

194 Harada N, Watanabe Y, Sato K, *et al*. Bone regeneration in a massive rat femur defect through endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold. *Biomaterials* 2014;**35**(27):7800–7810.

195 Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009;**4**(3):206–216.

196 Murphy JM, Fink DJ, Hunziker EB, Barry FP. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* 2003;**48**(12):3464–3474.

197 Horie M, Choi H, Lee RH, *et al*. Intra-articular injection of human mesenchymal stem cells (MSCs) promote rat meniscal regeneration by being activated to express Indian hedgehog that enhances expression of type II collagen. *Osteoarthritis Cartilage* 2012;**20**(10):1197–1207.

198 Wu L, Leijten JC, Georgi N, *et al*. Trophic effects of mesenchymal stem cells increase chondrocyte proliferation and matrix formation. *Tissue Eng Part A* 2011;**17**(9–10):1425–1436.

199 Wu L, Prins HJ, Helder MN, *et al*. Trophic effects of mesenchymal stem cells in chondrocyte co-cultures are independent of culture conditions and cell sources. *Tissue Eng Part A* 2012;**18**(15–16):1542–1551.

200 Aung A, Gupta G, Majid G, Varghese S. Osteoarthritic chondrocyte-secreted morphogens induce chondrogenic differentiation of human mesenchymal stem cells. *Arthritis Rheum* 2011;**63**(1):148–158.

201 Maumus M, Manferdini C, Toupet K, *et al*. Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. *Stem Cell Res* 2013;**11**(2):834–844.

202 Glorieux FH. Osteogenesis imperfecta. *Best Pract Res Clin Rheumatol* 2008;**22**(1):85–100.

203 Pereira RF, Halford KW, O'Hara MD, *et al*. Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Natl Acad Sci U S A* 1995;**92**(11):4857–4861.

204 Pereira RF, O'Hara MD, Laptev AV, *et al*. Marrow stromal cells as a source of progenitor cells for nonhematopoietic tissues in transgenic mice with a phenotype of osteogenesis imperfecta. *Proc Natl Acad Sci U S A* 1998;**95**(3):1142–1147.

205 Horwitz EM, Prockop DJ, Fitzpatrick LA, *et al*. Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999;**5**(3):309–313.

206 Horwitz EM, Prockop DJ, Gordon PL, *et al*. Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. *Blood* 2001;**97**(5):1227–1231.

207 Le Blanc K, Götherström C, Ringdén O, *et al*. Fetal mesenchymal stem-cell engraftment in bone after in utero transplantation in a patient with severe osteogenesis imperfecta. *Transplantation* 2005;**79**(11):1607–1614.

208 Niyibizi C, Li F. Potential implications of cell therapy for osteogenesis imperfecta. *Int J Clin Rheumatol* 2009;**4**(1):57–66.

209 Caplan AI. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J Cell Physiol* 2007;**213**(2):341–347.

210 Niyibizi C, Wang S, Mi Z, Robbins PD. The fate of mesenchymal stem cells transplanted into immunocompetent neonatal mice: implications for skeletal gene therapy via stem cells. *Mol Ther* 2004;**9**(6):955–963.

211 Li F, Wang X, Niyibizi C. Distribution of single-cell expanded marrow derived progenitors in a developing mouse model of osteogenesis imperfecta following systemic transplantation. *Stem Cells* 2007;**25**(12):3183–3193.

212 Otsuru S, Gordon PL, Shimono K, *et al*. Transplanted bone marrow mononuclear cells and MSCs impart clinical benefit to children with osteogenesis imperfecta through different mechanisms. *Blood* 2012;**120**(9):1933–1941.

213 Tran C, Damaser MS. Stem cells as drug delivery methods: application of stem cell secretome for regeneration. *Adv Drug Deliv Rev* 2015;**82–83**:1–11.

CHAPTER 13

# The role of glycogen synthase kinase-3 inhibitors on bone remodeling

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## 13.1 Overview of glycogen synthase kinase-3

The human skeleton is constantly renewing itself through a process called remodeling. Remodeling is achieved by the tightly coupled activities of bone-resorbing osteoclasts and bone-building osteoblasts, with the process regulated by osteocytes. Some bones, for example those of the craniofacial skeleton, are formed by intramembranous ossification, whilst most of the skeleton is formed through endochondral bone formation on to a cartilage scaffold [1]. In this chapter we describe the impact of the inhibition of glycogen synthase kinase-3 (GSK-3) on normal bone remodeling and within the context of bone disease. GSK-3 is a negative regulator of bone formation, with inhibition of GSK-3 activity resulting in the stimulation of bone formation. Preclinical data from *in vitro* and *in vivo* studies will be used to describe the impact of GSK-3 inhibition on bone loss in osteoporosis, multiple myeloma, and periodontal disease as well as in bone healing such as in fracture models. Key published data will be highlighted which suggest that this anabolic response can be attributed to alterations in mesenchymal stromal cell (MSC) functions, including proliferation, commitment, and differentiation of MSCs.

GSK-3 is a ubiquitously expressed serine/threonine kinase that inactivates glycogen synthase and was originally identified as an enzyme involved in the synthesis of glycogen [2,3]. More recently this multifunctional kinase has been shown to regulate additional cellular processes, including Tau protein and the canonical Wnt/β-catenin signaling cascade [4,5]. There are two isoforms of GSK-3 encoded by two genes, GSK-3α and GSK-3β, which in humans are located on chromosomes 19 and 3 respectively. At approximately 98%, the homology between GSK-3α (51 kDa) and GSK-3β (47 kDa) is particularly high in the catalytic domain, although overall homology is reduced to approximately 85% due to variability at the N- and C-termini [6]. GSK-3 is somewhat unusual in that its activity is generally high in resting cells and is inhibited upon cell stimulation through phosphorylation of $Ser^{21}$ in GSK-3α and $Ser^{9}$ in GSK-3β [3] either by insulin/phosphatidylinositide 3-kinase (PI3K)/AKT signaling [7] or through the disruption of the protein complex involved in canonical Wnt/

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β-catenin signaling [8]. There is also an alternatively spliced variant of GSK-3β encoding GSK-3β2, which contains a 13-amino-acid insert in the kinase domain and is reported to be neuron specific and to possess reduced phosphorylation activity toward Tau protein relative to GSK-3β [9,10]. In addition to glycogen metabolism, GSK-3 is involved in a variety of biological processes, including embryonic development, cell proliferation and apoptosis, insulin signaling, neuronal function, and oncogenesis. GSK-3 has been reported to have a number of putative targets, including Tau [11], microtubule-associated protein 1B [12], insulin receptor substrate 1 [13], β-catenin [14], cyclic AMP response element-binding protein [15], nuclear factor of activated T cells (NFAT) [16], CCAAT-enhancer binding protein (C/EBPα) [17], eukaryotic initiation factor 2B [18], and c-jun [19]. In addition to roles in insulin and Wnt/β-catenin signaling, GSK-3 also plays regulatory roles in the Hedgehog signaling pathway [20]. GSK-3 has been implicated in the pathogenesis of a range of diseases, including diabetes mellitus, cancer, neurodegenerative diseases, and bipolar affective disorders [20,21]. Consequently, GSK-3 has been considered an attractive drug target, and a range of GSK-3 inhibitors have been developed as potential treatment options for these diseases. The role of GSK-3 in these diseases has been reviewed extensively and falls outside the scope of this chapter [22–25].

## 13.2 The response of skeletal cells to glycogen synthase kinase-3 inhibitors *in vitro*

The effects of GSK-3 inhibition on osteoblastogenesis and osteoclastogenesis have been investigated using a range of GSK-3 inhibitors with variable potencies and selectivity profiles (Table 13.1).

GSK-3 inhibitors have been assigned as activators of the canonical Wnt/β-catenin signaling cascade and GSK-3 inhibition results in an induction of β-catenin transcriptional activities [33,34]. To confirm Wnt/β-catenin activity, investigators have used a number of *in vitro* methods. The three most commonly used methods are first, the stabilization of β-catenin within cell nuclei; second, an increase in dephosphorylated β-catenin levels; and third, the expression of a β-catenin/Transcription Factor (TCF)/lymphoid enhancer-binding factor (LEF)-responsive reporter construct; for example, TOP-flash [35] or one of its variants. The following section summarizes the role of GSK-3 in bone remodeling from studies that have utilized these GSK-3 inhibitors in *in vitro* assays in progenitor stem cell lines, preosteoblastic and osteoblast cell lines, and in primary skeletal cells or tissues. It is important to note that although GSK-3 inhibitors are often reported as GSK-3β inhibitors, the inhibitors included in this chapter demonstrate similar potency by both GSK-3α and GSK-3β. They should therefore be thought of as dual GSK-α/β inhibitors, and elucidating the precise roles of the individual isoforms in bone remodeling would be an interesting focus of future studies.

**Table 13.1** GSK3 inhibitors reported to affect bone remodeling *in vitro*.

| GSK-3 inhibitor | Parameter | GSK-3β potency (nM) | Reference |
|---|---|---|---|
| LiCl | $IC_{50}$ | 2000 | [26] |
| SB-216763 | $IC_{50}$ | 34.3[a] | [27] |
| SB-415286 | $IC_{50}$ | 77.5[a] | [27] |
| 6-bromoindirubin-3′-oxime | $IC_{50}$ | 5 | [28] |
| LY603281-31-8 | $IC_{50}$ | 1.3 | [29] |
| CHIR99021 | $IC_{50}$ | 6.7 | [30] |
| AR28/AZD2858 | $IC_{50}$ | 5 | [31] |
| AR79 | $K_i$ | 3.2 | [32] |

[a] Represents measurements of GSK-3α potency for SB-216763 and SB-415286. Potency is reported as equivalent at GSK-3β [27].

### 13.2.1 Lithium chloride

Lithium ions ($Li^+$) and lithium chloride (LiCl) have been reported to inhibit GSK-3 family members and produce effects in cellular systems consistent with GSK-3 inhibition [36,37]. These include, for example, the activation of glycogen synthase and subsequent insulin-mediated glycogen deposition demonstrated in rat adipocytes [38], and Wingless signaling reported in *Drosophila* [36]. GSK-3β was reported to be inhibited by $Li^+$ ($IC_{50}$, 2000 nM; Table 13.1) at three- to sevenfold lower concentrations than a range of other protein kinases, including MAPK-activated protein kinase 2 (MAPKAP-K2), p38-regulated/activated kinase (PRAK), and checkpoint kinase 2 [26]. In addition, glucocorticosteroids, including dexamethasone, have been shown to inhibit osteoblastic commitment and differentiation, resulting in the downregulation of the GSK-3 target gene *c-MYC*, increased GSK-3β kinase activity, and attenuation of GSK-3β $Ser^9$ inhibitory phosphorylation. However, the reduction in *c-MYC* expression has been reported to return to control levels by GSK-3 inhibition using LiCl [39].

Murine C3H10T1/2 cells are multipotent MSCs that have the ability to differentiate into osteoblasts, adipocytes, chondrocytes, and myoblasts. Nuclear translocation of β-catenin has been shown to be induced by 10 mM LiCl in a manner similar to Wnt3a in C3H10T1/2 cells [40]. Moreover, LiCl has been reported to increase TOP-flash activity in a time- and concentration-dependent manner, indicating that LiCl has the ability to stimulate TCF-dependent transcriptional activity [40,41]. For example, over a 24 h treatment period, 20 mM LiCl caused a 10-fold increase in TCF/LEF reporter activity relative to sodium chloride (NaCl) controls [41].

Similar results were obtained using human SaOS-2 osteoblastic cells in which exposure to LiCl led to a marked increase in nuclear β-catenin levels compared with controls [42]. In these studies the investigators demonstrated similar nuclear

accumulation of β-catenin in Wnt3a-treated murine MC3T3 preosteoblasts. The similarities observed between the distribution of β-catenin in the murine and human Wnt3a- and LiCL-treated osteoblastic cells led to the suggestion of a central role for GSK-3 inhibition and canonical Wnt/β-catenin signaling in osteoblasts [42].

The activation of endogenous Wnt/β-catenin signaling in C3H10T1/2 cells with LiCl led to an increase in the mRNA expression of the osteoblast marker alkaline phosphatase (ALP), which was of similar magnitude to that observed following bone morphogenetic protein 2 (BMP-2) stimulation, as well as an increase in alkaline phosphastase activity [40,43]. Runt-related transcription factor 2 (RUNX2) plays an essential role in osteoblastic differentiation and *RUNX2* knockout mice demonstrate a complete absence of bone due to the arrest of osteoblast maturation [44,45]. *RUNX2* has also been reported to be a Wnt/β-catenin/TCF target gene product [46]. RUNX2 protein expression was significantly increased in a time- and concentration-dependent manner following treatment with LiCl. Unlike BMP-2, however, these results indicated that LiCl has the ability to activate canonical Wnt/β-catenin signaling and accelerate early commitment to osteogenesis [40,43].

In addition, the canonical Wnt/β-catenin signaling may play an integral role in osteoblast differentiation and function. Using microarray technology, Vaes *et al.* investigated the expression of components of the Wnt signaling cascade during osteoblast differentiation induced by BMP-2 in murine C2C12 mesenchymal progenitor cells (MPCs) and MC3T3-E1 preosteoblasts [47]. Interestingly, the Wnt antagonists secreted frizzled related protein 2 and Wnt inhibitory factor 1, which were reported to be strongly upregulated during the late phase of osteoblast differentiation. Using human mesenchymal stromal cells the group further analyzed the effects of continuous activation of Wnt signaling by LiCl and observed that dexamethasone-induced osteoblast differentiation was reduced, as measured by expression of the osteoblast marker genes encoding ALP, osteocalcin, and osterix, as well as by the amount of calcium released. Taken together, the data indicated that endogenous expression of Wnt antagonists by osteoblasts provides a negative Wnt feedback loop that may be essential in regulating the final stages of osteoblast maturation [48].

The literature on the effects of glucocorticosteroids, and particularly dexamethasone, on osteoblast differentiation and maturation is conflicting. In MC3T3-E1 preosteoblast cells dexamethasone has been reported to cause a reduction in β-catenin levels and an inhibition in TCF/LEF-mediated transcriptional activity [49]. Abrogation of Wnt/β-catenin signaling is suggested to be a mechanism by which glucocorticosteroids inhibit the commitment-associated cell cycle during osteoblast differentiation. Dexamethasone-mediated reduction in β-catenin expression levels was attenuated by both GSK-3 inhibition with LiCl and PI3K/Akt inhibition with Wortmannin, leading the investigators to suggest that glucocorticosteroids may inhibit the osteoblast differentiation-related cell cycle through both Wnt/β-catenin-dependent and -independent mechanisms, and that PI3K/Akt/GSK-3/β-catenin/LEF axis inhibition and histone deacetylase stimulation may together mediate the effects of glucocorticosteroids on osteoblast differentiation [49]. (Wortmannin, a steroid metabolite of the fungus *Penicillium funiculo*sum, is a specific, covalent inhibitor of phosphoinositide 3-kinases.)

The effects of dexamethasone on canonical Wnt signaling were further investigated in primary human osteoblasts prepared from bone fragments from the femoral neck. Treatment with LiCl caused a 3.5-fold induction in TOP-flash reporter activity, comparable to effects observed following the use of Wnt3a-conditioned media [50]. Interestingly, the LiCl-mediated increase in Wnt/β-catenin signaling was not attenuated by dexamethasone, which led to the suggestion that dexamethasone affects canonical Wnt/β-catenin signaling either directly through GSK-3 or through regulation of an upstream component of the canonical Wnt cascade; for example, Dickkopf-1 [51].

As demonstrated so far, and throughout this chapter, the involvement of the canonical Wnt/β-catenin signaling cascade, including GSK-3 as a key regulator, has been generally accepted among the research community with regard to osteoblast differentiation. In contrast, however, the role of Wnt/β-catenin signaling in osteoclastogenesis remains controversial [52–54]. Osteoclast precursor cells, derived from the monocyte–macrophage lineage, fuse to form multinucleated cells that express tartrate-resistant acid phosphatase (TRAP). These cells function to resorb bone, and osteoclast differentiation is regulated by two cytokines: macrophage colony-stimulating factor (M-CSF) and receptor activator of nuclear factor-κB ligand (RANKL) [55].

To understand how osteoclast formation might be regulated by canonical Wnt/β-catenin signaling, Spencer *et al.* set up co-culture assays consisting of murine primary osteoblasts and spleen mononuclear cells. Treatment of these co-cultures with up to 20 mM LiCl over 12 days caused a significant, concentration-dependent reduction in the formation of multinucleated TRAP-positive cells relative to control cultured cells treated with NaCl. To provide evidence that the effects observed with LiCl treatment were mediated through Wnt/β-catenin signaling, similar experiments were performed to measure the effects of Wnt3a-conditioned medium on osteoclast formation *in vitro*. Over 12 days, treatment with Wnt3a-conditioned medium inhibited osteoclast differentiation to levels comparable to that observed with LiCl [42]. The investigators also demonstrated that RANKL mRNA and protein expression were completely inhibited in MC3T3-E1 and MG-63 osteoblastic cells following exposure to LiCl. While similar results were obtained in MC3T3 cells treated with Wnt3a, RANKL expression was not affected by exposure to the noncanonical Wnt ligand Wnt5a. These data appear to indicate that canonical Wnt/β-catenin signaling may inhibit osteoclastogenesis through the regulation of RANKL expressed by osteoblasts and suggest that changes in bone remodeling following modulation of the canonical Wnt pathway

may be due to effects on both bone formation and bone resorption [42].

Arioka *et al.* have investigated the effect of GSK-3 inhibitors on osteoclastogenesis using the murine osteoclast precursor RAW-D cells, isolated from RAW 264 cells [40]. RAW-D cells are responsive to RANKL and tumor necrosis factor-α and have been shown to differentiate to osteoclasts [56,57]. To determine the role of GSK-3 in osteoclastogenesis, RAW-D cells were incubated with RANKL for 4 days with or without GSK-3 inhibitors. Treatment with LiCl significantly reduced TRAP activity and the number of TRAP-positive multinucleated cells (MNCs) with >10 nuclei in a concentration-dependent manner, although treatment with Wnt3a produced contrasting effects, notably an increase in both TRAP activity and TRAP-positive MNC accumulation. The group then examined the effect of GSK-3 inhibitors on the expression of NFAT cytoplasmic 1 (NFATc1), which has been reported to be an essential transcription factor for osteoclast differentiation [58]. LiCl significantly inhibited NFATc1 mRNA and protein expression, whereas, once again, Wnt3a conversely increased their expression [40]. Thus, GSK-3 is thought to be involved not only in osteoblastogenesis but also in osteoclastogenesis [52–54] and the results reported by Arioka *et al.* [40] are consistent with previous reports [53,54]. However, the involvement of the canonical Wnt/β-catenin signaling pathway in osteoclastogenesis is still somewhat controversial, and the effects of GSK-3 inhibitors on osteoclastogenesis may be independent of Wnt/β-catenin signaling since Wnt3a was reported to stimulate osteoclast differentiation from precursor RAW-D cells.

The cellular responses to LiCl in both osteoblast and osteoclast differentiation are dependent on the cell culture conditions, serum concentration, the inducing factors used, and the timing of their administration [43].

### 13.2.2 SB-216763 and SB-415286

SB-216763 and SB-415286 are structurally distinct maleimide compounds that have been reported to inhibit GSK-3α *in vitro* with $IC_{50}$ values of 34.3 nM and 77.5 nM respectively in an ATP competitive manner. (Maleimides are chemical compounds with the formula $H_2C_2(CO)_2NH$. The name is a contraction of maleic acid and imide, the —C(O)NHC(O)— functional group.) These compounds were reported to inhibit GSK-3β with similar potency and were selective for GSK-3 over a panel of other protein kinases [27]. These compounds thus represent potent pharmacological tools with which the role of GSK-3 in cellular signaling can be further elucidated.

As with LiCl, SB-216763 treatment led to a significant reduction in phosphorylated β-catenin levels, increased nuclear stabilization of β-catenin and stimulated TCF/LEF transcriptional activity in C3H10T1/2 cells. SB-216763 also induced osteoblast differentiation, causing an increase in ALP expression, elevated R2 expression, and increased osteogenic mineralization relative to controls [40].

To further understand the role that GSK-3 plays in endochondral bone growth, Gillespie *et al.* investigated the effects of SB-415296 in e15.5 (15.5 days of gestation) mouse tibial organ cultures cultured for 6 days. Treatment with 10 μM SB-415286 led to a 31% increase in total tibial length. A similar trend was also observed using 10 μM SB-216763 [59]. This increase in tibial length did not affect the overall length of the epiphyseal growth plate of the treated tibia. However, treatment with SB-416286 did affect the organization and relative lengths of the component chondrocyte zones within the growth plate. The division between resting zone and proliferating zone chondrocytes was less distinct in SB-415286-treated tibias relative to controls, and the combined length of resting and proliferating zones was 13% longer in SB-415286-treated tibias relative to controls. In addition, SB-415286 treatment led to a 57% reduction in the length of hypertrophic zone of chondrocytes and a concomitant increase in the relative area of the tibia that had undergone mineralization. Together, these results suggest that inhibition of GSK-3 promotes long bone longitudinal growth by promoting bone formation [59].

The expansion of a capillary network and the provision of a functional microvasculature within the skeleton is essential for bone remodeling and fracture healing [60]. Vascular endothelial growth factor (VEGF) released from osteoblasts plays a significant physiological role as an intercellular mediator between osteoblasts and vascular endothelial cells. Furthermore, VEGF has been reported to be involved in trabecular bone formation and hypertrophic chondrocyte zone expansion in murine growth plates [61]. Using MC3T3-E1 preosteoblasts, Natsume *et al.* demonstrated that Wnt3a potentiated the release of VEGF stimulated by transforming growth factor-β (TGF-β) [62]. In addition, these investigators went on to show that TGF-β-stimulated VEGF release was markedly enhanced by treatment with SB-216763. Similar results were also observed following LiCl treatment and led to the suggestion that upregulation of TGF-β-stimulated VEGF synthesis following Wnt3a treatment is mediated through canonical Wnt/β-catenin signaling in osteoblasts. Kondo *et al.* demonstrated that Wnt3a significantly increased prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)-stimulated VEGF synthesis in MC3T3-E1 cells and also showed that SB-216763 markedly enhanced VEGF synthesis in response to $PGF_{2\alpha}$ stimulation to a similar degree [63]. Furthermore, and again in similar fashion to Wnt3a, SB-216763 had little effect on $PGF_{2\alpha}$-stimulated interleukin (IL)-6 synthesis, a potent bone resorptive agent [64]. Taken together, these studies led to speculation that the effects of canonical Wnt/β-catenin in bone metabolism is mediated, at least in part, by VEGF release from osteoblasts through fine modulation of signaling molecules, including TGF-β and $PGF_{2\alpha}$ [62,63].

Studies utilizing SB-415286 in the context of mechanical loading have provided interesting insights into the complex interactions of canonical Wnt/β-catenin signaling and additional signaling mechanisms in mesenchymal stromal cells. Mechanical strain has previously been reported to inactivate GSK-3 through $Ser^9$ phosphorylation within 30 min of the strain being

applied [65]. In C3H10T1/2 cells, mechanical loading has been reported to inhibit adipogenesis and, using 20 μM SB-415286 added to C3H10T1/2 cells cultured with the adipogenic supplements dexamethasone, insulin, and indomethacin, Sen *et al.* demonstrated that GSK-3 inhibitor treatment was able to prevent increases in peroxisome proliferator-activated receptor-γ (PPAR-γ) and adiponectin mRNA and protein expression in a manner similar to that induced by the application of strain. Similar results were obtained using LiCl [65]. In follow-up studies by the same group, small interfering RNA (siRNA) targeting GSK-3β prevented both strain-induced induction of β-catenin and an increase in cyclooxygenase-2 (COX2), a factor associated with increased osteoprogenitor phenotype [66]. siRNA knockdown of β-catenin blocked the mechanical strain-induced reduction of PPAR-γ and adiponectin expression, implicating Wnt/β-catenin signaling in strain-mediated inhibition of adipogenesis. In contrast, the effect of both mechanical strain-induced inhibition of GSK-3 and pharmacological inhibition using SB-415286 on the putative β-catenin target, COX2, remained unaffected by knockdown of β-catenin. SB-415286 treatment and mechanical strain caused nuclear accumulation of NFATc1, independent of β-catenin, and subsequent NFATc1 knockdown prevented the mechanical stimulation of COX2 expression, implying a role for NFATc1 signaling. Furthermore, GSK-3 inhibition led to the association of RNA polymerase II with the COX2 gene, suggesting the initiation of transcription [66]. In summary, these studies suggest that mechanical inhibition of GSK-3 induces activation of both canonical Wnt/β-catenin and NFATc1 signaling, limiting adipogenesis via Wnt signaling and promoting osteoblastic differentiation, in part, through NFATc1/COX2 [65,66]. Importantly, the inhibition of GSK-3 may affect MSC differentiation through the regulation of multiple downstream targets.

Similar to the results reported earlier with LiCl, treatment of RAW-D cells with SB-216763 significantly decreased both TRAP activity and the number of TRAP-positive MNCs in a concentration-dependent manner, indicating a potential role for canonical Wnt/β-catenin signaling in the regulation of osteoclastogenesis [40]. Interestingly, use of the other maleimide GSK-3 inhibitor SB415286 provided additional evidence of a potential effect of GSK-3 inhibition on osteoclastogenesis. Giant cell tumor of bone is a benign yet destructive neoplasm, with tumors consisting of mesenchymal fibroblast-like stromal cells, monocyte-like mononuclear cells of myeloid lineage, and characteristic osteoclast-like multinucleated giant cells. Notably, SB-415286 reversed the RANKL/osteoprotegerin (OPG) expression ratio and led to a significant reduction in the numbers of TRAP-positive MNCs, indicating a reduction in osteoclastogenic capacity. In contrast, recombinant human BMP-2 had the opposite effect, resulting in enhanced osteoclastogenesis. These data provide early evidence to suggest that targeting the giant cell tumor stromal cell may be worth investigating as a potential adjunct to existing antiresorptive strategies [67].

### 13.2.3 6-bromoindirubin-3′-oxime

The indirubin 6-bromoindirubin-3′-oxime (BIO) is a synthetic cell-permeable derivative of the natural product 6-bromoindirubin, a constituent of Tyrian purple, an indigo dye obtained from gastropod molluscs. Indirubins were discovered to be potent protein kinase inhibitors initially on cyclin-dependent kinases (CDKs) [68] and later on GSK-3 [69]. BIO retains enhanced selectivity for GSK-3 over CDKs [28].

COS-1 cells, fibroblast-like cells derived from African green monkey kidney, were exposed for 24 h to BIO, which caused a significant increase in the levels of unphosphorylated β-catenin accompanied by a modest increase in total β-catenin protein expression [28]. Wang *et al.* investigated MC3T3-E1 preosteoblasts maintained in an osteogenic medium containing β-glycerophosphate and ascorbic acid and used dexamethasone at high concentrations to model the effects of glucocorticosteroid administration on bone deterioration [70]. As with MC3T3-E1 cells, BIO treatment at 1 μM led to the stabilization of β-catenin protein in cell nuclei [71]. Treatment with 10 μM BIO was reported to attenuate dexamethasone-mediated inhibition of phosphorylated GSK-3$\beta^{Ser9}$ and nuclear β-catenin. BIO thus acted as a selective GSK-3 inhibitor in this cell line [70]. In addition, BIO induced a 100-fold increase relative to controls in TOP-flash TCF/LEF transcriptional activity in C3H10T1/2 cells following a 40 h treatment [41]. Krause *et al.* incubated human bone marrow-derived MSCs (BM-MSCs) prepared from iliac crest marrow aspirates under osteogenic conditions; BIO treatment resulted in elevated levels of β-catenin in cell nuclei and a reduction in cytoplasmic GSK-3β, consistent with an upregulation of Wnt/β-catenin signaling [72]. Indirubins have been described to interact with the aryl hydrocarbon receptor (AhR) [73]. To determine whether the observed BIO-induced β-catenin dephosphorylation and stabilization was mediated through an AhR-dependent effect, cell lines deficient either in AhR or AhR nuclear translocator (a co-transcriptional factor of AhR) were evaluated. BIO-induced β-catenin stabilization was maintained in both cell lines, demonstrating that its effects are mediated directly through GSK-3 rather than through an indirect, AhR-dependent pathway [28].

BIO treatment was also shown to increase Runx2 expression and the formation of mineralized nodules in MC3T3-E1 cells, assessed through von Kossa staining following dexamethasone-induced suppression, demonstrating that GSK-3 inhibition can protect osteoblasts against glucocorticosteroid-induced suppression of osteogenic differentiation [70]. Watanabe *et al.* showed that 1 μM BIO treatment stimulated osterix nuclear translocation and caused an upregulation of the mRNA transcription of Runx2, osterix, collagen type I, alpha 1 (Col1a1), and bone-specific ALP. In contrast, mRNA expression of osteocalcin, osteopontin, and dentin matrix protein 1, markers associated with osteoblast maturation and extracellular matrix mineralization, were reduced, suggesting that BIO treatment leads to increased numbers of cells with a relatively immature osteoblastic phenotype [71].

Human BM-MSCs were also incubated in osteogenic medium without dexamethasone; treatment with BIO at doses up to 200 nM led to increased ALP activity. However, doses of 400 nM and above caused ALP activity to return to control levels. OPG expression was increased in a concentration-dependent manner [72]. In extended cell culture experiments, BIO was reported to be unable to induce osteogenic mineralization in the presence of β-glycerophosphate and ascorbic acid but in the absence of dexamethasone. When osteogenic media was supplemented with dexamethasone, however, BIO increased mineralization in a concentration-dependent manner, as assessed through Alizarin red S staining. BIO was demonstrated to enhance dexamethasone-dependent mineralization when used either as an 8 day pretreatment or when present throughout a 17 day culture period, indicating that Wnt/β-catenin stimulation is required to induce an osteoprogenitor phenotype in human BM-MSCs [72].

### 13.2.4 LY603281-31-8

LY603281-31-8 is an orally bioavailable GSK-3 inhibitor that exhibits selectivity for GSK-3β over a panel of other kinases [29] (Table 13.1). The effects of LY603281-31-8 on MSCs have been investigated in two main studies [74,75]. Treatment of murine C3H10T1/2 cells for 24 h led to increased β-catenin protein expression in whole-cell lysates and a two- to sevenfold increase in TCF/LEF transcriptional activity following transient transfection with the TOP-flash reporter [74]. LY603281-31-8 was therefore reported to be a potent activator of canonical Wnt/β-catenin signaling.

Although being reported to exert biphasic effects on a number of bone-specific marker genes, LY603281-31-8 treatment caused increased mRNA expression of collagen-α1 (I) and -α1 (V), bone sialoprotein, Runx2, and osteocalcin after 24 h of treatment relative to controls. In addition, ALP mRNA expression and enzyme activity were increased significantly in C3H10T1/2 cells after 24 h, and the investigators reported a reduction in cell proliferation [74].

In addition to evaluating markers of osteoblast differentiation, Kulkarni *et al.* also investigated the effects of LY603281-31-8 on markers of adipogenic differentiation. LY603281-31-8 treatment led to significant reductions in the expression of CEBP-α (which encodes CCAAT-enhancer-binding protein-α), GPD1 (which encodes glycerol-3-phosphate dehydrogenase), lipoprotein lipase (LPL), fatty acid binding protein 4, and PPAR. These results are consistent with LY603281-31-8 causing enhanced osteogenic commitment and osteoblastic differentiation and suppression of adipogenic differentiation [74].

Kulkarni *et al.* also investigated the effects of LY603281-31-8 on MPCs isolated from rat bone marrow stromal cells (BMSCs). In *ex vivo* studies, osteoprogenitor cells were identified by colony-forming unit–fibroblast (CFU-F) assays followed by ALP staining [75]. Rats were subjected to oophorectomy (removal of the ovaries) and treatment with parathyroid hormone (PTH) or LY603281-31-8. Oophorectomy led to a significant 18% reduction in CFU-F colony numbers. While this was further decreased following treatment with PTH, treatment with LY603281-31-8 increased the number of CFU-F colonies to 125% of oophorectomized control levels. In addition, while oophorectomy led to a significant reduction in the ratio of ALP-positive CFU-F colonies, treatment with PTH or LY603281-31-8 significantly increased the production of putative osteoblasts [75]. In summary, LY603281-31-8 acts as a selective GSK-3 inhibitor to induce osteoblast differentiation, leading to an increase in bone formation, further supporting a role for canonical Wnt/β-catenin signaling in osteoblastogenesis.

### 13.2.5 CT99021/CHIR99021

The aminopyrimidine GSK-3 inhibitor CT99021/CHIR99021 is a potent GSK-3 inhibitor that has demonstrated 500- to >10 000-fold selectivity over a panel of other kinases [30] (Table 13.1). This compound is extremely interesting, in that, in addition to its reported effects on osteoblastogenesis, CT99021/CHIR99021 is routinely used in combination with a mitogen-activated protein kinase inhibitor, PD0325901, to maintain mouse embryonic stem cells (mESCs) in a ground state of pluripotency [76]. A ground state of pluripotency is a basal proliferative state that is free of epigenetic restriction and has minimal requirements for extrinsic stimuli.

CT99021/CHIR99021 has been recommended for use as a potent and selective GSK-3 inhibitor and is reported to completely prevent the phosphorylation of a number of GSK-3 substrates, including *N-Myc* downstream-regulated gene 1 [77].

Canonical Wnt signaling cues have also been reported to specify the differentiation of germ layers and multipotent stem cells into mesodermal cells [78]. Kanke *et al.* have reported a strategy to efficiently differentiate both mESCs and mouse induced pluripotent stem cells toward the osteoblast lineage by sequentially treating the cells with four small molecules, CT99021/CHIR99021, cyclopamine, SAG (a small-molecule agonist of the Sonic hedgehog-Smoothened (Shh-Smo) signaling pathway), and a helioxanthin-derivative -(4 methoxyphenyl)pyrido[4′,3′:4,5]thieno[2,3-*b*] pyridine-2-carboxamide [79].

Using CT99021/CHIR99021 Bennett *et al.* reported that Wnt/β-catenin signaling was able to stimulate osteoblastic differentiation and inhibit adipogenic differentiation of bipotential mouse ST2 BMSCs. Following incubation in a medium containing β-glycerophosphate and L-ascorbic acid, CT99021/CHIR99021 treatment led to elevated ALP activity and increased osteogenic mineralization as assessed by Alizarin red staining. CT99021/CHIR99021 also inhibited adipogenesis as visualized by Oil red O staining [80]. Although the effects of CT99021/CHIR99021 were consistent with canonical Wnt/β-catenin signaling inducing osteoblast differentiation directly, Bennett *et al.* identified that, with GSK-3 playing a regulatory role in a number of signaling pathways, there is the potential that CT99021/CHIR99021 stimulates osteoblastogenesis through alternate signaling mechanisms. To address this, activation of canonical Wnt/β-catenin signaling was achieved through retroviral

infection of Wnt1, Wnt10b, or dominant-stable β-catenin in ST2 cells. These cell lines demonstrated increased ALP activity, accelerated osteoblast differentiation, and increased mineralization when cultured in osteogenic media. Control ST2 cells demonstrated relatively low levels of ALP activity and mineralization under these conditions, and background levels of osteoblastic differentiation were not increased either by the presence of a dominant negative transcription factor 7-like 2 (TCF7L2) construct or through the activation of noncanonical Wnt signaling using Wnt5a. Wnt5a also demonstrated no appreciable effect on adipogenesis in ST2 cells, whereas activation of the canonical pathway with Wnt10b or Wnt1 completely inhibited adipocyte differentiation. Similar results were also observed in C3H10T1/2 cells [80].

However, contrasting data have been reported by Frith *et al.* [81]. Using stromal precursor antigen-1-positive human bone-marrow-derived MPCs in a continuous perfusion microbioreactor array (MBA) platform, enzyme-labelled fluorescence 97 (ELF97) was used to assess ALP activity as a marker of early osteogenic differentiation. Treatment with CT99021/CHIR99021 led to a significant reduction in ELF97 expression that correlated with an increase in DNA content. In additional studies this group investigated whether similar results would be obtained with MPCs grown in standard culture formats. Similar to the results obtained with the MBA platform, ELF97 expression was increased when MPCs were cultured in the presence of osteogenic supplements. However, ELF97 expression was strongly inhibited by treatment with CT99021/CHIR99021. CT99021/CHIR99021 was profoundly inhibitory of ALP activity at all concentrations above 1 μM. In additional studies the group investigated the effects on the later stages of osteoblast differentiation using Alizarin red S staining to determine the extent of mineral deposition after 21 days. Osteogenic medium induced the formation of Alizarin red deposits across the majority of the culture surface, and this was almost completely abolished with CT99021/CHIR99021 treatment. In contrast, however, determination of gene expression at 7 days showed that expression of the osteoblastic markers Runx2, Msh homeobox protein 2 and distal-less homeobox 5 was significantly upregulated in MPCs treated with CHIR99021 relative to controls. However, ALP expression remained significantly inhibited by CT99021/CHIR99021 [81].

### 13.2.6 AR28 (AZD2858), AR79, and AZ13282107

AZD2858, AR79, and AZ13282107 are potent GSK-3 inhibitors derived from three chemical series (Table 13.1). The compounds exhibit similar potency on GSK-3β and GSK-3α and cause the stabilization of β-catenin in cell nuclei [32].

In studies by Gambardella *et al.*, AR28, hereafter referred to as AZD2858, was shown to increase functional β-catenin protein expression in a concentration-dependent manner following 24 h of treatment of C3H10T1/2 cells [31]. In further studies designed to understand whether AZD2858 could promote an increase in MPC number, murine bone marrow cells were exposed to AZD2858 and the numbers of CFU-Fs, colony-forming unit–adipocytes (CFU-As), and colony forming unit–osteoblasts (CFU-Os) were determined. Relative to controls, treatment of bone marrow cells with AZD2858 led to significant increases in the number of CFU-Fs and in the numbers of MPCs with adipogenic (CFU-A) and osteogenic (CFU-O) potential. To determine whether AZD2858 was able to stimulate differentiation of MPCs towards the osteogenic lineage, bone marrow cells were cultured for 14 days in a medium supplemented with β-glycerophosphate, L-ascorbic acid, and dexamethasone. Significant increases in ALP and osteocalcin gene expression were observed following exposure to AZD2858, suggesting that GSK-3 inhibition promoted differentiation towards the osteogenic lineage. The numbers of CFU-As were determined using a 4 week assay comprising a 2 week expansion phase in basal medium followed by a 2 week differentiation phase using adipogenic medium supplements. AZD2858 was added either during the expansion phase or the differentiation phase and it was reported that a significant increase in CFU-As was only observed when the GSK-3 inhibitor was added during the expansion phase. There was no increase in CFU-A number when AZD2858 was added during the differentiation phase. Exposure to AZD2858 also caused no change in the expression of the adipogenic marker genes LPL and PPAR-γ. Thus, these data suggested that GSK-3 inhibition using AZD2858 increased the number of MPCs with both osteogenic and adipogenic potential, but drove differentiation only toward the osteogenic lineage [31]. The data are consistent with observations from rat BM-MSCs, indicating a relative increase in the numbers of putative osteoblast cells generated following treatment with the GSK-3 inhibitor LY603281-31-8 [75].

The ability of AZD2858, AR79, and AZ13282107 to drive the commitment of human adipose-derived stem cells toward osteoblastogenesis was investigated by assessment of the osteoblast-specific markers osterix and the protein transcriptional co-activator with PDZ-binding motif (TAZ). Similar to Runx2, osterix is a master regulator of skeletal development and has been reported to be essential for osteoblast differentiation and bone formation, exemplified by the absence of osteoblast differentiation and maturation in osterix-null mice [82]. TAZ has been reported to modulate the activity of Runx2 and PPAR-γ during MSC lineage commitment [83], and has recently been reported as an integral component of the canonical Wnt/β-catenin pathway [84,85]. Protein expression of both osterix and TAZ was increased following treatment with AZD2858, AR79, and AZ13282107 relative to vehicle controls [32]. In follow-up studies the three GSK-3 inhibitors were evaluated for their ability to drive osteogenic mineralization in human adipose-derived stromal cells (ADSCs) as assessed by Alizarin red S staining. Over 25 days of cell culture, incubation with AZD2858, AR79, or AZ13282107 led to a marked enhancement in osteogenic mineralization relative to controls. Importantly, it should also be noted that the human ADSCs exposed to GSK-3 inhibitors were maintained in a basal medium without the

addition of osteogenic supplements. Taken together, these findings indicate that AZD2858, AR79, and AZ13282107 stimulate osteoblastogenesis and enhance osteogenic differentiation of human ADSCs [32].

In C3H10T1/2 MPCs, human BMSCs isolated from the bone marrow from the femoral head, and primary human osteoblast-like cells isolated from hip arthroplasty patients, AZD2858 was demonstrated to increase nuclear β-catenin translocation and to increase stable dephosphorylated β-catenin levels [41,86]. AZD2858 treatment led to a dose-dependent increase in TCF/LEF transcriptional activity with 2.5 μM AZD2858 causing an approximate 1000-fold increase relative to controls, an effect comparable to the magnitude of effect induced by Wnt3a (100 ng/mL), 100-fold greater than that of LiCl, and 10-fold greater than that induced by BIO [41]. Following the removal of the AZD2858 stimulus the Wnt/β-catenin response remained elevated for several hours before declining, leading to the suggestion that canonical Wnt/β-catenin activation may be reversible.

The effect of AZD2858 on adipogenesis of BMSCs was evaluated. When BMSCs were cultured in adipogenic medium, a large increase in Oil Red O staining was observed. However, AZD2858 treatment abrogated Oil Red O staining in a concentration-dependent manner, indicating that AZD2858 inhibits adipogenic differentiation in BMSCs. After 14 days in adipogenic medium, human ADSCs were stained for the adipocyte markers fatty acid binding protein 5 (FABP5) and BODIPY (borondipyrromethene, a lipid probe) and the Hoechst nuclear stain. Approximately 50–60% of human ADSC controls demonstrated positive staining for both FABP5 and BODIPY. Consistent with the results obtained with BMSCs, AZD2858 significantly inhibited adipogenesis in human ADSCs [41].

BMSCs were initially cultured for up to 3 weeks in the presence of β-glycerophosphate, L-ascorbic acid, and dexamethasone. AZD2858 caused a significant reduction in ALP expression in a concentration-dependent manner. Osteogenic mineralization, assessed by von Kossa staining, was also reduced in response to administration of AZD2858. Under standard dexamethasone-induced osteogenic conditions, these data indicated that Wnt/β-catenin activation with AZD2858 significantly inhibits ALP activity and does not stimulate significant osteogenic mineralization *in vitro* [41]. Work by other groups [72,87] has demonstrated that canonical Wnt/β-catenin signaling may increase early osteogenic differentiation of BMSCs. Therefore, Cook *et al.* cultured BMSCs in media containing β-glycerophosphate and L-ascorbic acid but without dexamethasone. Under these conditions, small but significant increases in ALP activity were observed following treatment with low doses of AZD2858. However, ALP activity was reduced when AZD2858 concentrations were increased to 0.5 μM. In addition, although AZD2858 had no effect on von Kossa staining, osteogenic mineralization was increased in a concentration-dependent manner as assessed by Alizarin red S staining when dexamethasone was removed from osteogenic medium. This suggests that, when dexamethasone is absent, AZD2858-stimulated Wnt/β-catenin signaling can elevate osteoblastic differentiation in a manner that is dependent on BMSC number [41].

Taken together, work from a variety of *in vitro* studies utilizing a range of GSK-3 inhibitors has indicated that pharmacological GSK-3 inhibition results in commitment of MSCs to osteoblastic differentiation and mineralization. The canonical Wnt/β-catenin signaling pathway is complex, being regulated by multiple Wnt ligands, Wnt receptors, and numerous soluble inhibitors [88]. In addition, the variety of MSCs and osteoblastic cells studied, combined with differences in species, study protocols, and the timing and duration of GSK-3 inhibitor treatment have led to some variability in the results obtained from different research groups. However, as detailed below, the indications of a pro-bone formation phenotype derived from *in vitro* assays translates *in vivo* to serum bone turnover biomarker changes consistent with increased bone formation and reduced resorption.

## 13.3 Bone anabolism through inhibition of glycogen synthase kinase-3 *in vivo*

The development of GSK-3β knockout mice and treatment with small molecule inhibitors of GSK-3 have consistently shown changes in bone remodeling resulting in an overall increase in bone mass in rodents *in vivo*. Heterozygous GSK-3β-deficient (GSK-$3\beta^{+/-}$) mice grow normally without disorders in major organs and without gross skeletal abnormalities. However, radiographs and three-dimensional computed tomography (CT) imaging identified an increase in bone mass and cortical and trabecular bone volumes in femurs taken from GSK-$3\beta^{+/-}$ mice compared with wild-type littermates [89,90]. These changes in bone mass were accompanied by increases in trabecular bone volume and cortical thickness, as well as enhanced bone formation rates, in GSK-$3\beta^{+/-}$ mice compared with wild-type mice. In a partial bone defect model using femoral burr holes, accelerated bone healing was measured in GSK-$3\beta^{+/-}$ compared with wild-type mice [90]. In GSK-$3\beta^{+/-}$ mice, accelerated bone formation was associated with increased protein expression of β-catenin and the Wnt target genes cyclin D1, *C-MYC* and *RUNX2* [90]. Runx2 is known to be a critical transcription factor in the differentiation of osteoblasts. Thus, enhanced expression of Runx2 in GSK-$3\beta^{+/-}$ mice is thought to stimulate bone anabolism by regulating osteoblastogenesis *in vivo*. In fact, heterozygous deficient $Runx2^{+/-}$ mice showed delayed closure of the skull fontanelles and hypoplasia of the clavicles due to impaired bone formation. The fontanelles are an anatomical feature of the infant human skull comprising any of the soft membranous gaps between the cranial bones that make up the calvaria of a fetus or an infant. (The calvarium is the portion of the skull bones forming the roof of the cranial cavity.) Fontanelles allow for rapid stretching and deformation of the neurocranium, as the brain expands faster than the surrounding bone can grow. $Runx2^{+/-}$ mice have a similar phenotype to that of the human disease cleidocranial dysplasia [89]. Cleidocranial dysplasia is a

generalized skeletal disease so named from the collarbone (cleido-) and cranium deformities that people with it often have. These abnormalities in the fontanelles and clavicles were less in dual GSK-3$\beta^{+/-}$ Runx2$^{+/-}$ mice. Additionally, LiCl administration restored both the fontanelle and clavicle abnormalities in the Runx2$^{+/-}$ mice. These studies inducing a genetic deficiency in GSK-3β have clearly shown GSK-3β to be a negative regulator of bone formation. Inhibition of GSK-3β not only stimulates bone formation in mice but also rescues bone abnormalities induced by defects in the pathways involved in osteoblastogenesis.

Several rodent studies using small molecule inhibitors of GSK-3 have corroborated these findings by demonstrating increased bone mass/volume assessed by peripheral quantitative CT/micro-CT and bone formation assessed by dynamic or static histomorphometry analysis following compound treatment in mature animals. Bone anabolism was seen after only 2–6 weeks of repeat dosing in mice or rats with LiCl, BIO, AZD2858, AR79, and AZ13282107 (Table 13.2).

Repeat dosing with LiCl, BIO, or AZD2858 increased trabecular number and thickness and bone volume/trabecular volume (BV/TV) ratio in normal healthy mice. In rats, increases in bone mass following chronic AZD2858 treatment also resulted in stronger bones as measured by biomechanical bending tests [86].

Bone anabolism observed following inhibition of GSK-3 could be attributed to the regulation of multiple pathways involved in osteoblastogenesis and mature osteoblast function, such as:

- commitment of undifferentiated cells to the osteoblast lineage with a reduction in adipogenesis through canonical Wnt signaling, as demonstrated by genetic modification of Wnt signaling *in vivo* [80,91];
- proliferation of preosteoblasts, as suggested by the increased number of osteoblasts per total bone area with GSK-3 inhibitor treatment *in vivo* [31,91];
- reduced osteoblast apoptosis, as observed following GSK-3 inhibitor treatment of calvarial cultures *in vitro* [91].

Alterations in the control of bone resorption may also play a role in the overall increase in bone mass observed with GSK-3 inhibition. The regulation of bone anabolism versus resorption will be expanded upon in subsequent sections of this chapter.

### 13.3.1 Functional Wnt/β-catenin responses in *Xenopus laevis* model systems

*Xenopus laevis* is the scientific (Latin) name of the African clawed frog, which is commonly used as an organism for studying developmental biology, cell biology, toxicology, and neuroscience. Maternal Wnt activity is required for dorsal axis formation in *X. laevis* embryos [28]. Axis duplication has been assessed using *in vivo* model systems of *X. laevis* to determine whether compounds have the ability to selectively activate a functional canonical Wnt/β-catenin response [99]. Cook *et al.* demonstrated that injection of AZD2858 into *X. laevis* embryos caused an axis duplication event, visible as Y-shaped dorsal axes and secondary neural tube formation. The severity of axis duplication increased with increasing concentration of AZD2858, visualized by a higher rate of occurrence of duplicated eyes [41]. Similar effects can be generated via the manipulation of alternative signaling pathways such as BMPs [100]. Therefore, chordin expression was assessed in order to confirm that the effects were due to the specific activation of Wnt/β-catenin. Chordin is a marker of the Spemann organizer [101,102] and is specific for canonical Wnt/β-catenin activation. The Spemann organizer is the organizer for gastrulation in vertebrates. Gastrulation is a phase early in the embryonic development of most animals during which the single-layered blastula is reorganized into a trilaminar ("three-layered") structure known as the gastrula. These three germ layers are known as the ectoderm, mesoderm, and endoderm. Injection of AZD2858 led to the development of distinct secondary regions of chordin expression on the ventral side in 95% of the embryos injected. No second regions were identified in control embryos. AZD2858 induces specific canonical Wnt/β-catenin responses *in vitro* and *in vivo* [41]. In addition to chordin, the canonical Wnt/β-catenin pathway can induce dorsal genes such as the direct Wnt target siamois [103]. *Siamois*, a *Xenopus* zygotic homeobox gene with strong dorsalizing activity, is expressed in the dorsal–vegetal organizer known as the Nieuwkoop center. RT-PCR analysis of animal cap explants treated with either BIO or LiCl resulted in induction of both chordin and siamois [28].

### 13.3.2 Progenitor cell involvement in bone anabolism *in vivo*

Although GSK-3 inhibitors have demonstrated an ability to stimulate preosteoblast differentiation to osteoblasts *in vitro*, there is limited information on the role of GSK-3 and stromal progenitor cells in the bone anabolic response *in vivo*. This section summarizes data in healthy rodents.

#### 13.3.2.1 Alteration in osteoblast progenitor cell lineage *in vivo*

In 8-week-old rats, repeat dosing with AZD2858 daily for 14 days produced an increase in bone mass that was characterized by an increase in both total and cortical bone mineral content, trabecular bone mineral density (BMD), and a dramatic increase in bone formation rate of 480% (Figure 13.1).

In addition, AZD2858 produced a significant increase in force at failure in bone bending tests, denoting increased bone strength. Enhanced bone mass with increased trabecular thickness and number was also seen after 14 days of treatment with AZD2858 in mice [31]. In this study, colony-forming units taken from the bone marrow were counted after 3 and 14 days of dosing with AZD2858; a rise in both CFU-Os and CFU-As was noted after 3 days but not 14 days, compared with vehicle control mice. At day 14, only an increase in CFU-Fs was seen. Thus, a transient early rise in progenitors with potential to differentiate to osteoblasts or adipocytes was observed prior to the increase in bone mass. In tibias, mature osteoblast numbers were increased after 14 days of treatment with AZD2858 with a reduction in the bone

**Table 13.2** Summary of bone changes with GSK3 inhibitors in animal models.

| Animal model | GSK3 inhibitor | Duration of dosing with GSK3 inhibitor | Bone findings with GSK3 inhibitor compared with vehicle group | Reference |
|---|---|---|---|---|
| *Healthy rodents* | | | | |
| Naive mice | LiCl | Daily for 1 month | Increase in trabecular number and thickness, 35% increase in BV/TV and osteoblast number | [91] |
| Naive mice | BIO | Every 48 h for 6 weeks | Increased trabecular number, 18% increase in BV/TV and 30% serum ALP | [92] |
| Naive mice | AZD2858 | Daily for 14 days | Increase in trabecular number and thickness and 50% increase in BV/TV | [31] |
| Naive rats | AZD2858 | Daily for 14 days | Increase in bone mass, rate of bone formation and bone strength, and plasma osteocalcin | [86] |
| Naive rats | AZD2858, AR79, AZ13282107 | Daily for 14–28 days | Increase in bone mass and changes in bone turnover biomarkers (increased P1 NP and decreased TRACP-5b) | [32] |
| *Models of bone loss* | | | | |
| Ovariectomy-induced osteopenia in 6-month-old rats | 603281-31-8 | Daily for 2 months post-ovariectomy | Increased BMD, restored bone volume, normalized marrow adiposity, and increased vertebral biomechanical strength. Increased mRNA expression levels of osteoblast differentiation/function | [74,75] |
| Glucocorticoid-induced bone loss in rats | BIO | Weekly for 6 weeks with methylprednisolone | Prevented the loss in BMD and biomechanical strength and the reduction in bone formation induced by glucocorticoid | [70] |
| Osteopenia in SAMP6 mice | LiCl | Daily for 4 weeks | Increased BV/TV, osteoblast number, and mineral surface | [91] |
| Methotrexate (Mtx)-induced bone loss in rats | BIO | Daily for 5 days with Mtx | Prevented the reduction in BV/TV and trabecular number induced by Mtx. Reduced $TRAP^+$ cells in bone | [93] |
| Osteolysis in 5TGM1 myeloma mice | LiCl | Daily from tumor inoculation | Increased BMD, trabecular volume, and osteoblasts. Reduced $TRAP^+$ cells in bone | [94] |
| Osteolytic lesions induced by xenograft myeloma in SCID mice | BIO | Peri-tumoral injection every 2 days after onset of plasmacytomas | Improved bone area, trabecular thickness, and bone fraction and quality at the bone–tumor interface | [92] |
| Alveolar bone loss in *P. gingivalis*-infected mice | SB-216763 | 1 day prior to infection and then every other day | Prevented alveolar bone loss and reduced systemic inflammation | [95] |
| Periapical bone lesions in rat apical periodontitis | LiCl | Daily after lesion: | | [96] |
| | | from day 7 to 21 | Decreased bone lesions and $TRAP^+$ osteoclasts with increased BV/TV and osteoblast number/surface | |
| | | from day 0 to 28 | No change in bone lesions | |
| *Fracture healing* | | | | |
| Fracture (mouse tibia with intramedullary fixation) | LiCl | Dosed 2 weeks before fracture | Reduced bone density and volume at the fracture site | [97] |
| | | Dosed from 4 days after fracture | Increased bone density and volume at the fracture site | |
| Fracture (rat femur with intramedullary fixation) | AZD2858 (AR28) | Dosed for 3 weeks after fracture | Increased callus area, BMD, BMC, and strength in the fractured limb | [98] |

BV/TV: bone volume/trabecular volume ratio; BMC: bone mineral content.

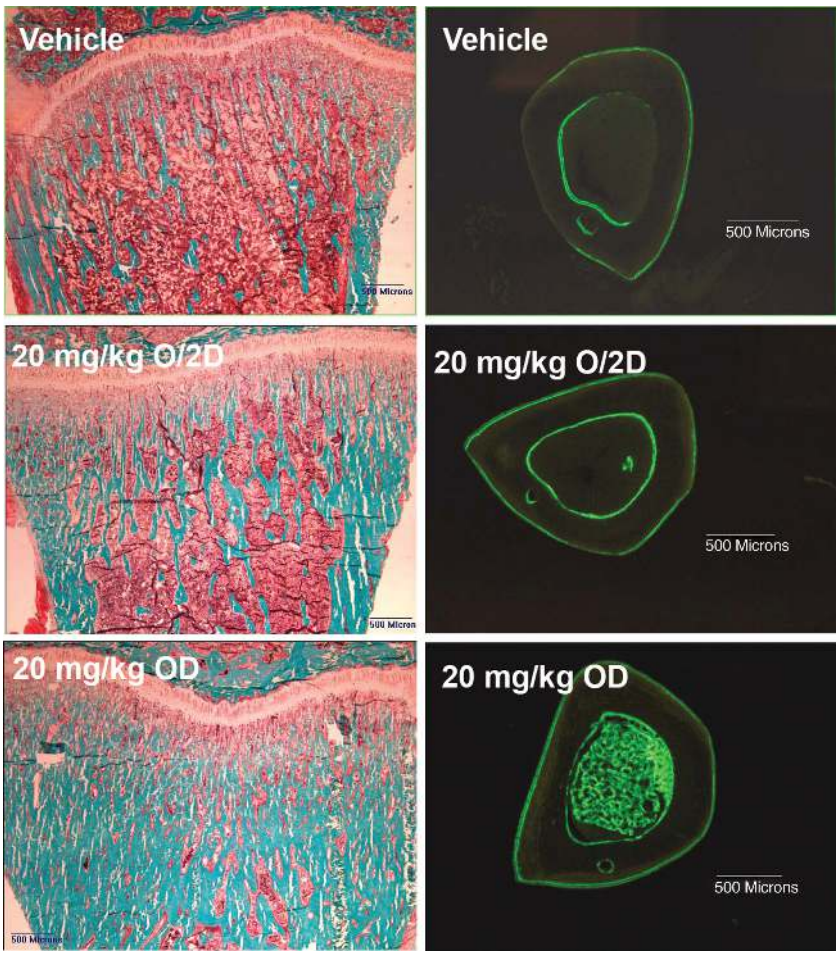



**Figure 13.1** Images showing increased bone formation with AZD2858 treatment in rats. Histology of the proximal tibia metaphysis and mid-diaphysis is shown. Left panels show Masson-Trichrome stained longitudinal sections from the proximal tibia (bright-field microscope pictures). Blue staining indicates bone tissue and red staining indicates soft tissues, including the growth plate and marrow cavity. Right panels show transverse sections of mid-diaphysis double labeled with calcein, a green fluorescent bone-staining dye. (OD; once daily, O/2D; once every 2 days). (See color plate section.) *Source*: Marsell 2012 [86], pp. 619–627. Reproduced with permission from Elsevier.

marrow area occupied by the lipid content of adipocytes at 3 and 14 days. These data suggested that treatment with AZD2858 results in an early wave of MPCs that commit to the osteogenic but not the adipocyte lineage *in vivo* [31]. This provides *in vivo* evidence that stromal progenitor cells commit to the osteoblast lineage and likely contribute to bone anabolism following chronic treatment of GSK-3 inhibitor in rodents. We are not aware of any other *in vivo* studies that have investigated progenitor cell changes after GSK-3 inhibitor treatment in normal healthy rodents.

Biomarkers of osteoblast activity/bone formation, including ALP, osteocalcin, and the N-terminal propeptide of type 1 procollagen (P1NP), are released from mature osteoblasts during bone formation. GSK-3 inhibitors (BIO, AZD2858, AR79, and AZ13282107) increase the levels of these biomarkers in the systemic circulation in rodents [32,86,92], thus providing further evidence that there is increased osteoblast activation and/or number when GSK-3 enzyme activity is reduced *in vivo*.

### 13.3.3 Alteration in bone resorption *in vivo*

The relative impact of GSK-3 inhibition on bone resorption in normal or diseased bone is not well understood *in vivo*. A change in the balance between the rate of bone formation and resorption can alter serum biomarkers, and three different GSK-3 inhibitors have shown an increase in the serum bone formation biomarker P1NP with a concomitant reduction in the bone resorption marker tartrate-resistant acid phosphatase 5b (TRACP5b) *in vivo* [32], thus suggesting an uncoupling of bone formation and bone resorption following inhibition of GSK-3 in rats. However, enhanced bone turnover was demonstrated in a separate study in which the collagen breakdown product C-telopeptide (CTX) was increased with AZD2858 administration in rats [86]. These contrasting findings on biomarkers of bone resorption may be attributed to differences in the magnitude and duration of GSK-3 inhibition achieved *in vivo*. For example, daily dosing with AZD2858 20 mg/kg per day significantly increased serum CTX, whereas intermittent dosing once every other day with 20 mg/kg did not alter CTX levels, even though both dosing regimens produced a significant increase in bone mass at 14 days [86].

Murine colony-forming units of granulocytes and macrophages, which are precursors to osteoclasts, either rose at 3 days or were the same as those in vehicle-treated mice at 14 days of AZD2858 treatment, although a significant increase in

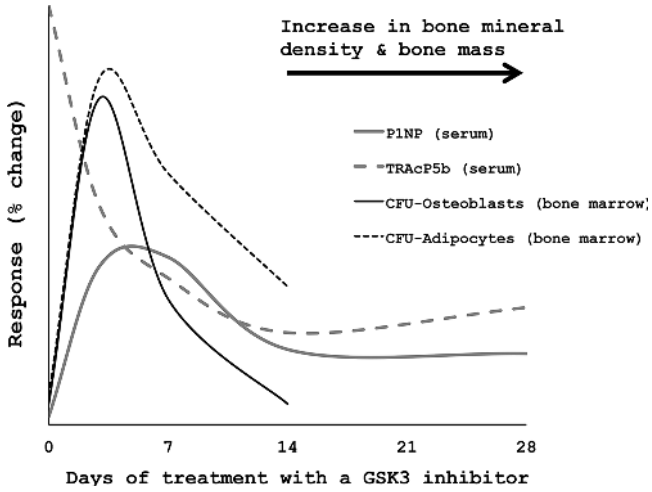



**Figure 13.2** Schematic illustrating the temporal changes in bone marrow progenitor cells, serum bone turnover biomarkers, and bone mass in rodents following daily treatment with GSK-3 inhibitors.

osteoclasts on the endocortical surface was measured in the AZD2858-treated mice compared with vehicle-treated mice [31]. To date, GSK-3 inhibition *in vivo* has shown an overall rise in bone mass, probably as a result of enhanced osteoblast numbers/function that outweighed any increase in bone resorption. The conflicting literature on the role of GSK-3 in osteoclastogenesis and mature osteoclast function and the limited *in vivo* data in this area make it difficult to determine whether GSK-3 inhibition directly or indirectly affects bone resorption. Thus, this area of biology warrants further investigation.

In summary, it is interesting to note that in adult rodents there is a rapid mobilization of bone marrow stromal progenitor cells and systemic biomarkers of bone turnover within a few days of dosing with GSK-3 inhibitors, which then leads to discernable mineralized bone within weeks in otherwise healthy, uninjured bone (see schematic in Figure 13.2).

This bone anabolic response is rapid even in adult rodent bones where bone turnover has slowed due to the natural reduction in bone growth with age. Bone mass is increased globally within the body and particularly within trabecular bone in long bones such as the tibia and femur. The mechanisms behind this change in bone turnover with GSK-3 inhibitors are not localized to a specific type of bone, and this is not unexpected due to the ubiquitous distribution of GSK-3 within osteoblasts and progenitors.

## 13.4 Impact of glycogen synthase kinase-3 inhibition in bone disease

Inhibition of GSK-3 may be beneficial in treating osteoporosis and osteolytic diseases by driving the maturation of osteoblasts, and thus osteogenesis. Several animal models of bone loss have been used to explore the biology of GSK-3 in bone anabolism and resorption, with some evidence of alterations in progenitor cell lineage toward osteoblastogenesis over adipogenesis and chondrogenesis (summarized in Table 13.2).

### 13.4.1 Osteopenia and osteoporosis

Osteopenia can be induced by estrogen depletion following oophorectomy or chronic dosing with glucocorticosteroids *in vivo*. Oophorectomy in rats leads to significant bone loss and increased marrow adiposity, which is associated with changes in the expression of multiple genes, including a decrease in markers of osteogenesis and chrondrogenesis and an increase in markers of adipogenesis and lipid metabolism [74,75]. Long-term administration of LY603281-31-8 in oophorectomized rats restored bone loss and reversed bone marrow adiposity. Analysis of marrow stromal cells measured by the number of CFU-Fs from rats treated with LY603281-31-8 showed an increase in total CFU-Fs and CFU-F/ALP$^+$ cells compared with oophorectomized control rats. These results suggest that the reversal in bone loss with GSK-3 inhibition is caused by progenitor cell commitment to the osteoblast lineage with a concomitant reduction in adipogenesis in osteopenic rats [75].

It is well known that long-term treatment with glucocorticosteroids suppresses the formation of bone which can lead to osteoporosis and an increased risk of fractures. Animal models have therefore been developed to recapitulate some of these bone changes. Male rats treated with methylprednisolone for 6 weeks demonstrated reductions in bone weight, global mineral content, and femoral mineral density [70]. In addition, bone strength and bone formation rate were reduced in methylprednisolone-treated rats. BIO, administered for six consecutive weeks, attenuated the loss of bone density and biomechanical strength and prevented the reduction in bone formation rate induced by chronic glucocorticosteroid treatment. The precise mechanisms by which BIO altered bone turnover in this model are not known, but *in vitro* studies using BIO suggest that increases in osteoblast differentiation, maturation, and viability may be involved. GSK-3 inhibitors such as BIO can also protect against glucocorticosteroid-induced apoptosis in osteoblastic cell lines *in vitro* [70]. In addition, increases in marrow adipocytes and osteoclasts were observed with glucocorticosteroid treatment, and these responses were reversed with BIO, thus suggesting that inhibition of GSK-3 may be regulating adipogenesis and/or osteoclastogenesis induced by glucocorticosteroids.

LiCl was found to alter the balance of adipocytes and osteoblasts in bone marrow progenitor cells obtained from femurs in a murine dexamethasone-induced bone loss model. Daily injection of dexamethasone for 5 weeks caused a reduction in trabecular bone volume, bone thickness, and BMD in mice [104]. A rise in adipocyte numbers in the trabecular bone marrow space of these dexamethasone-treated mice was also seen. Studies using BMSCs from the femurs of dexamethasone-treated mice demonstrated a reduced ability to differentiate into osteoblasts and an upregulation of adipocyte markers

compared with BMSCs from normal mice. LiCl treatment of the BMSCs from dexamethasone-treated mice produced an increase in the osteoblastogenic markers osterix and osteocalcin with a dramatic reduction in the adipocyte markers C/EBP, adipocyte protein 2, and Glut4 in the presence of BMP-2. Thus, LiCl, through the activation of Wnt signaling, regulated progenitor cell differentiation pathways of dexamethasone-treated BMSCs to favor differentiation to osteoblasts over adipocytes. Additional studies with GSK-3 inhibitor treatment *in vivo* are required to determine the translatability of these *in vitro* BMSC findings of preference for osteoblastogenesis and bone formation over adipogenesis in glucocorticosteroid-induced osteoporosis models.

GSK-3 inhibition has also been investigated in a murine model of senescence-induced osteopenia. The senescence-accelerated mouse (SAMP6) has many features of senile osteoporosis, showing an accelerated osteoporosis phenotype due to reduced osteoblastogenesis [105]. Treatment of SAMP6 mice for 4 weeks with LiCl increased bone volume and osteoblast number and reduced marrow adiposity [91]. No changes in osteoclast numbers were observed. In this model, GSK-3 inhibition could again be diverting stromal bone marrow cells from the adipocyte lineage toward the osteoblast lineage, thus favoring bone formation.

Osteoporosis is also observed in low-density lipoprotein receptor-related protein 5 (LRP5)-deficient mice. LRP5 transduces the Wnt signal via the canonical pathway, leading to β-catenin accumulation in the nucleus and stimulation of gene transcription, resulting in bone production. One month of treatment of $LRP5^{-/-}$ mice with LiCl increased bone volume and bone formation rates *in vivo* and was associated with a concomitant increase in Wnt-response genes in bone marrow cells. LiCl was shown to activate intracellular Wnt signaling, independently of LRP5, which caused osteoblastogenesis in $LRP5^{-/-}$-deficient cells *in vitro* and increased bone formation in $LRP5^{-/-}$ mice *in vivo* [91].

### 13.4.2 Methotrexate-induced bone loss

Long-term chemotherapy with agents such as methotrexate is associated with an increase in the prevalence of bone-related complications, such as reduced BMD, osteoporosis, marrow adiposity, and an increased fracture risk. In rodent models, methotrexate decreases trabecular bone volume and increases marrow fat accumulation. As well as myelosuppression, methotrexate alters the balance of osteogenesis and adipogenesis in the bone marrow with short-term (5 days) and longer term (intermittent over 6 weeks) dosing regimens in rats [93]. This was demonstrated in *in vitro* differentiation assays with BMSCs by a decreased potential for osteogenic differentiation manifested by decreased expression of Runx2 and osterix and an increased commitment to adipogenesis manifested as increased PPAR-γ levels [93]. Methotrexate was shown to reduce Wnt and β-catenin signaling in bone and bone marrow cells (BMSCs) after 5 days of dosing in rats [106]. Administration of BIO concomitantly with methotrexate over 5 days attenuated the decrease in bone volume and trabecular number and abrogated the increase in adipocyte number, but not fat volume, induced by methotrexate treatment. In addition, the reduction in Wnt/β-catenin gene expression in metaphyseal bone induced by methotrexate was prevented by concurrent BIO treatment. Analysis of BMSC populations *in vitro* following methotrexate treatment demonstrated a reduction in mineralization potential that was reversed by BIO. However, BIO did not have an effect on the methotrexate-induced adipogenesis at day 6. Thus, in this model of methotrexate-induced bone loss, BIO treatment preserved bone loss through activation of Wnt signaling, which led to osteogenesis but only partially abrogated adipogenesis. This suggests that other non-Wnt pathways could be involved in the regulation of adipocyte differentiation and fat deposition following methotrexate treatment.

Long-term treatment with drugs such as glucocorticosteroids and methotrexate can cause bone loss in patients. Preclinical models, which reproduce some of these bone findings, demonstrate changes in stromal progenitor cell differentiation with a preference for adipogenesis over osteogenesis *in vivo*. GSK-3 inhibitors were able to prevent drug-induced bone loss and stimulate bone formation via activation of the Wnt/β-catenin signaling pathway.

### 13.4.3 Fracture healing

Fracture healing is a complex process involving many different cell types, including stromal progenitor cells. Initially, during fracture repair, MSCs accumulate at the site of bone injury, proliferate, and differentiate during two healing processes: intramembranous ossification and endochondral ossification. Intramembranous ossification involves bone formation directly from committed osteoprogenitor cells and undifferentiated MSCs in the periosteum, leading to hard callus formation. In endochondral ossification, MSCs differentiate to chondrocytes, producing a cartilaginous matrix that then becomes calcified and replaced by bone. Since GSK-3 inhibitors can upregulate β-catenin and stimulate progenitor cell commitment to the osteoblastic lineage they are likely to impact on fracture healing. LiCl or AZD2858, administered after induction of a fracture, enhanced fracture healing, as demonstrated by increases in bone volume, mass, and strength as well as by the rate of healing of fractures in rodents [97,98]. Improved fracture healing by LiCl acts in part via β-catenin signaling, as demonstrated using conditional β-catenin null mutations in a mouse fracture model. LiCl increased β-catenin levels in tissues at fracture sites, which was thought to stimulate osteoblast differentiation and new bone formation [97]. AZD2858 administration produced an increase in BMD and content in bone fracture calluses after only 2–3 weeks of treatment in a rat fracture model [98]. Histological analysis of the time course of bone healing showed a more rapidly healing process without the preformation of cartilage tissue, suggesting GSK-3 inhibition may prevent differentiation of chondrocytes and enable preferential differentiation of MSCs to osteoblasts [98].

The timing of intervention with GSK-3 inhibitors during the different stages of fracture repair may be critical for successful healing [107]. In contrast to the enhancement in healing observed with LiCl treatment that started 4 days after the fracture, pretreatment of mice with LiCl for 2 weeks prior to induction of fractures prevented healing of the fractures. At the fracture site, even though an increase in β-catenin expression was seen, there was evidence of undifferentiated MSCs with this "prophylactic" LiCl treatment. This finding suggests an inability of GSK-3 inhibitors to stimulate chondrogenesis and/or osteoblastogenesis during the early stages of fracture healing [97]. Although β-catenin stabilization is thought to be an important signaling process in fracture repair, it has been shown that changes in β-catenin levels in both gain-of-function and loss-of-function mutations in mice can prevent fracture healing *in vivo*. The cellular localization of β-catenin is critical as stabilization of β-catenin in specific osteoblast cells in conditional gain-of-function mice led to enhanced bone healing [97]. Thus, controlled stimulation of β-catenin during the initial stages of fracture healing with respect to timing and cellular locations is important in ensuring successful fracture healing through differentiation of MSCs from osteoblastic precursors to mature osteoblasts without altering chondrogenesis.

### 13.4.4 Multiple myeloma-associated bone disease

Multiple myeloma is a malignant neoplasm of mature plasma cells in the bone marrow, one result of which is the occurrence of osteolytic (bone loss) lesions throughout the skeleton. These osteolytic lesions develop in the bone as a consequence of complex changes in cytokines in the bone marrow, including increased RANKL and decreased OPG, osteoclastogenesis, and a reduction in MSC differentiation to osteoblasts. The malignant plasma cells impair osteogenic differentiation of MSCs through cell contact or release of soluble factors, including IL-7, hepatocyte growth factor, IL-3, and Wnt inhibitors. These changes in the skeletal microenvironment result in an overall increase in bone loss with impairment of bone formation, bone pain, and an increased risk of fractures. A variety of DNA polymorphisms, including the *GSK-3β* gene, have been linked to bone disease in people with myeloma [108]. GSK-3 is thought to be a prosurvival kinase in myeloma primary cells or cell lines. The GSK-3β inhibitors thiadiazolidinone, BIO, SB-216763, and SB-415286 are effective at inducing apoptosis of these malignant cells [92,109,110].

In addition to reducing myeloma cell survival, GSK-3 inhibitors may also reduce the osteolytic lesions in multiple myeloma. Blockade of GSK-3 could bypass the inhibition of the Wnt cascade in multiple myeloma induced by soluble Wnt inhibitors such as Dickkopf factor (DKK1) and thereby improve osteoblast maturation and osteogenesis. LiCl administration in the 5TGM1 murine myeloma model prevented osteolysis as measured by an increase in BMD, trabecular volume, and osteoblast numbers. A reduction in $TRAP^+$ cells and tumor burden within the bone was also seen [94]. Peritumoral administration of BIO after induction of plasmacytomas (malignant plasma cell tumors) in mice increased bone area, trabecular thickness, bone perimeter, bone fraction, and quality at the bone–tumor interface [92]. The improvement in bone quality could be due to a direct effect on osteogenesis rather than a reduction in tumor survival as the rate of growth of the tumor was similar in control versus BIO-treated groups. However, as BIO caused an increase in necrotic tumor tissue and LiCl reduced tumor burden in the bone, a direct effect of GSK-3 inhibition on tumor cell burden cannot be excluded [92,94]. As bone resorption is a prominent feature in myeloma, further studies are warranted to understand the ability of GSK-3 inhibitors to modulate osteoclastogenesis as well as osteoblastogenesis in these preclinical models of the disease.

### 13.4.5 Periodontal disease

Bone loss in the jaw from oral diseases such as periodontitis and periapical infections is common in adult humans. The bacterium *Porphyromonas gingivalis* initiates an innate immune system response mainly through activation of the toll-related receptors TLR-2 and TLR-4 (see Chapter 33). This produces an inflammatory response and periodontal bone loss. The exact mechanisms involved in this bone loss are unknown. *In vitro P. gingivalis* lipopolysaccharide (also known as endotoxin) reduces GSK-3β phosphorylation, β-catenin nuclear localization, and ALP activity in BM-MSCs, resulting in a reduction of osteogenic potential [96]. Osteogenesis can be restored by the addition of Wnt3a to the BM-MSC cultures, inhibition of GSK-3β, and activation of Wnt signaling.

Periapical lesions were formed by occlusal exposure of the pulp in both mandibular first molars for 4 weeks in rats. Rats treated with LiCl from days 7 to 21 after induction of the lesion had reduced periapical lesions and improved BV/TV ratios compared with control groups [96]. In addition, an increase in osteoblast numbers and expression of the osteoblast differentiation markers Runx2, osterix, osteocalcin, and bone sialoprotein in the periapical region were observed, as well as a reduction in $TRAP^+$ osteoclasts adjacent to periapical bone [96]. In contrast, continuous dosing for 28 days did not alter apical lesion and significantly prevented bone formation. In a mouse model of *P. gingivalis* infection, systemic pretreatment with SB-216763 prevented pathogen-induced bone loss on the buccal surface of the maxillary molar teeth [95]. In addition, this GSK-3 inhibitor reduced neutrophil infiltration and IL-17 levels in gingival tissues and decreased the level of circulating inflammatory cytokines, thus suggesting that GSK-3 inhibition may restore osteoblastogenesis via Wnt pathway activation and reduce osteoclastogenesis via a reduction in IL-17 in periodontal *P. gingivalis* infection.

### 13.4.6 Clinical findings with lithium

The only clinical trials that have investigated the effect of GSK-3 inhibition on bone turnover have been with lithium. Conflicting reports from these clinical studies have shown either no effect or

a decreased or an increased bone turnover. An example of a positive effect on BMD was evaluated in people on lithium carbonate therapy for psychiatric disorders for more than 1 year. These individuals had a 4.5–7.5% increase in BMD in the spine, femoral neck, and trochanter of the hip compared with control individuals [111]. Reduced levels of serum ALP, C-telopeptide, and osteocalcin in the lithium recipients also suggested an overall lowering of bone turnover.

The increased risk of fractures in osteoporotic patients has led to the development of new therapies for bone loss. The relationship between the long-term use of lithium and fracture risk has been investigated in a number of clinical studies. An observational study investigating the impact of chronic lithium treatment on the risk of any fracture demonstrated a reduced risk of fracture (odds ratio 0.75) in individuals treated with lithium over age- and gender-matched controls [112]. In a case–control study, the use of lithium was associated with a decreased risk of fractures that was not altered by the cumulative duration of lithium use [113]. However, robust clinical data are limited owing to the lack of randomized controlled trials in this field and the lack of GSK-3 inhibitors with suitable properties for use in humans.

## 13.5 Summary

It is widely recognized that key developmental signaling pathways, including those involving bone morphogenetic proteins, fibroblast growth factor, and wingless (Wnt), have a critical role to play in MSC biology, with a complex interplay of signaling through these pathways coordinating both proliferation and lineage specification [114]. However, although much has been elucidated about the roles of different signaling mechanisms on MSC fate, many conclusions have been confounded by the fact that the cellular response is critically dependent on microenvironmental parameters such as cell density at the onset of differentiation [115], the timing of exposure to inductive signals, and the impact of autocrine and paracrine signaling [116–118].

Clearly, GSK-3 inhibitors stimulate bone formation in both healthy bones and in a variety of disease models of bone loss, such as myeloma, and in fracture healing in rodents. Bone anabolism is observed with GSK-3 inhibition in the healthy bones and fractured bone within the same animal, demonstrating the ubiquitous role that GSK-3 plays in regulating bone turnover [98]. GSK-3 inhibitors have been shown to alter progenitor cell commitment in bone to an osteoblastic/ALP$^+$ phenotype, suggesting that bone anabolism induced by GSK-3 inhibitors is likely to be caused by increased osteoblastogenesis. The main signaling pathway for this anabolic effect is likely to be activation of Wnt signaling and upregulation of nuclear β-catenin. In addition, it is possible that GSK-3 inhibitors may have a dual role in stimulating bone formation and altering bone resorption. Uncoupling of bone anabolism and resorption has been demonstrated in healthy rodents [32], and a reduction in osteoclast number has been observed in several models using GSK-3 inhibitors, including oophorectomized rodents, a rodent model of myeloma, and a rodent model of periodontal disease. However, there is conflicting literature on the role of GSK-3 in bone resorption, osteoclastogenesis, and osteoclast function, particularly from *in vitro* studies that unfortunately cannot recapitulate the complex microenvironment of intact bone.

Depending on the complexity of the microenvironment of the bone during disease, GSK-3 can alter commitment of stromal progenitor cells to different lineages: osteoblasts, adipocytes, or chondrocytes. In models of osteopenia, such as that seen in oophorectomized animals or in recipients of glucocorticosteroids, the reversal in bone loss following GSK-3 inhibition was caused by progenitor cell commitment to the osteoblast lineage with a concomitant reduction in commitment to adipogenesis. For healing of bone fractures, increased osteoblastogenesis with GSK-3 inhibitors occurred but was associated with a reduction in chondrogenesis in some circumstances. In periodontal infection due to *P. gingivalis*, GSK-3 inhibition restored osteoblastogenesis as well as reduced osteoclastogenesis. The commitment of progenitor cells to different lineages was probably due to the ability of GSK-3 inhibitors to modulate many different intracellular pathways, including those of Wnt, AKT, and SMAD, but was also likely affected by the specific bone microenvironment altered by the disease. Further studies are required to understand the interplay between GSK-3 inhibition and these pathways in stromal progenitor cell commitment *in vivo*.

The timing of intervention with GSK-3 inhibitors may be critical in achieving a successful therapeutic intervention, as illustrated in the healing process of fractures and periodontal bone lesions. Prophylactic or early intervention with lithium during the healing process delayed bone healing. In contrast, enhanced bone healing was observed when dosing started at 4 days and 7 days after the lesion was induced in the fracture and periodontal models respectively. The mechanisms behind these differences – for example, Wnt- or non-Wnt-mediated mechanisms – is currently unclear, but inhibition of chondrogenesis during the early phase of fracture healing is known to cause delayed healing.

Translating these preclinical findings with GSK-3 inhibitors to the clinic is key to understanding the role of GSK-3 in human bone disease and could potentially provide new treatments for bone diseases. However, the impact of GSK-3 inhibitors on other cell types and tissues has potential to limit utility in the clinic owing to possible side effects with continuous dosing [119]. Intermittent dosing may allow stimulation of bone anabolism while allowing other tissues to recover from the potential adverse effects of GSK-3 inhibition. Intermittent dosing was effective in models of bone loss due to myeloma or to glucocorticosteroid administration and in healthy rats *in vivo*.

Small molecules may be an attractive alternative to cell replacement approaches to amplify progenitor cell pools or to direct their differentiation by targeting specific signaling pathways. Moreover, they can be used to understand basic mechanisms regulating progenitor cell self-renewal and differentiation.

Elucidating how Wnts act as instructive cues for the recruitment, maintenance, and maturation of MSCs and their differentiated progenies is of significant interest given the potential use of these cells in regenerative medicine [31].

In conclusion, our data are compatible with inhibition of GSK-3 acting on proliferation and commitment of MSCs with osteo- and adipogenic potential and which is driven to osteogenic differentiation at the expense of adipogenic differentiation. Our data also highlight the complex network of responses taking place with time and occurring in different cell types at specific stages of commitment. The work draws attention to the powerful effect that amplifying progenitor cells *in vivo* may have on bone mass. Further work is required to exclude the possibility of a reduction in progenitor cell numbers following prolonged treatment. It will also be important to determine the optimal dose and schedule of administration and to dissect the molecular events mediating the feedback mechanism to guarantee a prolonged and sustained effect of GSK-3 inhibitors as a bone anabolic treatment.

## References


1 J. H. Bassett and G. R. Williams. The molecular actions of thyroid hormone in bone. *Trends Endocrinol Metab* 2003;**14**: 356–364.

2 N. Embi, D. B. Rylatt, and P. Cohen. Glycogen synthase kinase-3 from rabbit skeletal muscle. Separation from cyclic-AMP-dependent protein kinase and phosphorylase kinase. *Eur J Biochem* 1980;**107**:519–527.

3 P. Cohen and S. Frame. The renaissance of GSK3. *Nat Rev Mol Cell Biol* 2001;**2**:769–776.

4 H. Eldar-Finkelman. Glycogen synthase kinase 3: an emerging therapeutic target. *Trends Mol Med* 2002;**8**:126–132.

5 R. S. Jope and G. V. Johnson. The glamour and gloom of glycogen synthase kinase-3. *Trends Biochem Sci* 2004;**29**:95–102.

6 J. R. Woodgett. Molecular cloning and expression of glycogen synthase kinase-3/factor A. *EMBO J* 1990;**9**:2431–2438.

7 D. A. Cross, D. R. Alessi, P. Cohen, *et al.* Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature* 1995;**378**:785–789.

8 B. W. Doble and J. R. Woodgett. GSK-3: tricks of the trade for a multi-tasking kinase. *J Cell Sci* 2003;**116**:1175–1186.

9 F. Mukai, K. Ishiguro, Y. Sano, and S. C. Fujita. Alternative splicing isoform of tau protein kinase I/glycogen synthase kinase 3β. *J Neurochem* 2002;**81**:1073–1083.

10 K. Saeki, M. Machida, Y. Kinoshita, and S. C. Fujita. Glycogen synthase kinase-3β2 has lower phosphorylation activity to tau than glycogen synthase kinase-3β1. *Biol Pharm Bull* 2011;**34**:146–149.

11 B. R. Sperber, S. Leight, M. Goedert, and V. M. Lee. Glycogen synthase kinase-3β phosphorylates tau protein at multiple sites in intact cells. *Neurosci Lett* 1995;**197**:149–153.

12 F. R. Lucas, R. G. Goold, P. R. Gordon-Weeksand, and P. C. Salinas. Inhibition of GSK-3β leading to the loss of phosphorylated MAP-1B is an early event in axonal remodelling induced by WNT-7a or lithium. *J Cell Sci* 1998;**111**(Pt 10):1351–1361.

13 H. Eldar-Finkelman and E. G. Krebs. Phosphorylation of insulin receptor substrate 1 by glycogen synthase kinase 3 impairs insulin action. *Proc Natl Acad Sci U S A* 1997;**94**:9660–9664.

14 M. J. Hart, R. de los Santos, I. N. Albert, *et al.* Downregulation of β-catenin by human Axin and its association with the APC tumor suppressor, β-catenin and GSK3β. *Curr Biol* 1998;**8**:573–581.

15 C. J. Fiol, J. S. Williams, C. H. Chou, *et al.* A secondary phosphorylation of CREB341 at Ser129 is required for the cAMP-mediated control of gene expression. A role for glycogen synthase kinase-3 in the control of gene expression. *J Biol Chem* 1994;**269**:32187–32193.

16 C. R. Beals, C. M. Sheridan, C. W. Turck, *et al.* Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science* 1997;**275**:1930–1934.

17 S. E. Ross, R. L. Erickson, N. Hemati, and D. A. MacDougald. Glycogen synthase kinase 3 is an insulin-regulated C/EBPα kinase. *Mol Cell Biol* 1999;**19**:8433–8441.

18 G. I. Welsh, C. M. Miller, A. J. Loughlin, *et al.* Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Lett* 1998;**421**:125–130.

19 E. Nikolakaki, P. J. Coffer, R. Hemelsoet, *et al.* Glycogen synthase kinase 3 phosphorylates Jun family members *in vitro* and negatively regulates their transactivating potential in intact cells. *Oncogene* 1993;**8**:833–840.

20 F. Takahashi-Yanaga. Activator or inhibitor? GSK-3 as a new drug target. *Biochem Pharmacol* 2013;**86**:191–199.

21 G. V. Rayasam, V. K. Tulasi, R. Sodhi, *et al.* Glycogen synthase kinase 3: more than a namesake. *Br J Pharmacol* 2009;**156**:885–898.

22 P. Cohen and M. Goedert. GSK3 inhibitors: development and therapeutic potential. *Nat Rev Drug Discov* 2004;**3**:479–487.

23 L. Meijer, M. Flajolet, and P. Greengard. Pharmacological inhibitors of glycogen synthase kinase 3. *Trends Pharmacol Sci* 2004;**25**:471–480.

24 H. Eldar-Finkelman and A. Martinez. GSK-3 inhibitors: preclinical and clinical focus on CNS. *Front Mol Neurosci* 2011;**4**:32.

25 S. Berg, M. Bergh, S. Hellberg, *et al.* Discovery of novel potent and highly selective glycogen synthase kinase-3β (GSK3β) inhibitors for Alzheimer's disease: design, synthesis, and characterization of pyrazines. *J Med Chem* 2012;**55**:9107–9119.

26 S. P. Davies, H. Reddy, M. Caivano, and P. Cohen. Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem J* 2000;**351**:95–105.

27 M. P. Coghlan, A. A. Culbert, D. A. Cross, *et al.* Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem Biol* 2000;**7**:793–803.

28 L. Meijer, A. L. Skaltsounis, P. Magiatis, *et al.* GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem Biol* 2003;**10**:1255–1266.

29 T. A. Engler, J. R. Henry, S. Malhotra, *et al.* Substituted 3-imidazo [1,2-*a*]pyridin-3-yl-4-(1,2,3,4-tetrahydro-[1,4]diazepino-[6,7,1-*hi*]indol-7-yl)pyrrole-2,5-diones as highly selective and potent inhibitors of glycogen synthase kinase-3. *J Med Chem* 2004;**47**: 3934–3937.

30 A. S. Wagman, K. W. Johnson, and D. E. Bussiere. Discovery and development of GSK3 inhibitors for the treatment of type 2 diabetes. *Curr Pharm Des* 2004;**10**:1105–1137.

31 A. Gambardella, C. K. Nagaraju, P. J. O'Shea, *et al.* Glycogen synthase kinase-3α/β inhibition promotes *in vivo* amplification

of endogenous mesenchymal progenitors with osteogenic and adipogenic potential and their differentiation to the osteogenic lineage. *J Bone Miner Res* 2011;**26**:811–821.

32 P. S. Gilmour, P. J. O'Shea, M. Fagura, *et al*. Human stem cell osteoblastogenesis mediated by novel glycogen synthase kinase 3 inhibitors induces bone formation and a unique bone turnover biomarker profile in rats. *Toxicol Appl Pharmacol* 2013;**272**: 399–407.

33 C. Hartmann. A Wnt canon orchestrating osteoblastogenesis. *Trends Cell Biol* 2006;**16**:151–158.

34 D. G. Monroe, M. E. McGee-Lawrence, M. J. Oursler, and J. J. Westendorf. Update on Wnt signaling in bone cell biology and bone disease. *Gene* 2012;**492**:1–18.

35 M. Molenaar, M. van de Wetering, M. Oosterwegel, *et al*. XTcf-3 transcription factor mediates β-catenin-induced axis formation in *Xenopus* embryos. *Cell* 1996;**86**:391–399.

36 V. Stambolic, L. Ruel, and J. R. Woodgett. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol* 1996;**6**:1664–1668.

37 P. S. Klein and D. A. Melton. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci U S A* 1996;**93**: 8455–8459.

38 K. Cheng, S. Creacy, and J. Larner. "Insulin-like" effects of lithium ion on isolated rat adipocytes. II. Specific activation of glycogen synthase. *Mol Cell Biochem* 1983;**56**:183–189.

39 E. Smith, G. A. Coetzee, and B. Frenkel. Glucocorticoids inhibit cell cycle progression in differentiating osteoblasts via glycogen synthase kinase-3β. *J Biol Chem* 2002;**277**:18191–18197.

40 M. Arioka, F. Takahashi-Yanaga, M. Sasaki, *et al*. Acceleration of bone regeneration by local application of lithium: Wnt signal-mediated osteoblastogenesis and Wnt signal-independent suppression of osteoclastogenesis. *Biochem Pharmacol* 2014;**90**: 397–405.

41 D. A. Cook, S. W. Fellgett, M. E. Pownall, and P. G. Genever. Wnt-dependent osteogenic commitment of bone marrow stromal cells using a novel GSK3β inhibitor. *Stem Cell Res* 2014;**12**:415–427.

42 G. J. Spencer, J. C. Utting, S. L. Etheridge, *et al*. Wnt signalling in osteoblasts regulates expression of the receptor activator of NFκB ligand and inhibits osteoclastogenesis *in vitro*. *J Cell Sci* 2006;**119**: 1283–1296.

43 G. Bain, T. Muller, X. Wang, and J. Papkoff. Activated β-catenin induces osteoblast differentiation of C3H10T1/2 cells and participates in BMP2 mediated signal transduction. *Biochem Biophys Res Commun* 2003;**301**:84–91.

44 P. Ducy, R. Zhang, V. Geoffroy, *et al*. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. *Cell* 1997;**89**:747–754.

45 T. Komori, H. Yagi, S. Nomura, *et al*. Targeted disruption of Cbfa1 results in a complete lack of bone formation owing to maturational arrest of osteoblasts. *Cell* 1997;**89**:755–764.

46 T. Gaur, C. J. Lengner, H. Hovhannisyan, *et al*. Canonical WNT signaling promotes osteogenesis by directly stimulating *Runx2* gene expression. *J Biol Chem* 2005;**280**:33132–33140.

47 B. L. Vaes, K. J. Dechering, E. P. van Someren, *et al*. Microarray analysis reveals expression regulation of Wnt antagonists in differentiating osteoblasts. *Bone* 2005;**36**:803–811.

48 R. Baron and G. Rawadi. Targeting the Wnt/β-catenin pathway to regulate bone formation in the adult skeleton. *Endocrinology* 2007;**148**:2635–2643.

49 E. Smith and B. Frenkel. Glucocorticoids inhibit the transcriptional activity of LEF/TCF in differentiating osteoblasts in a glycogen synthase kinase-3β-dependent and -independent manner. *J Biol Chem* 2005;**280**:2388–2394.

50 K. Ohnaka, M. Tanabe, H. Kawate, *et al*. Glucocorticoid suppresses the canonical Wnt signal in cultured human osteoblasts. *Biochem Biophys Res Commun* 2005;**329**:177–181.

51 K. Ohnaka, H. Taniguchi, H. Kawate, *et al*. Glucocorticoid enhances the expression of dickkopf-1 in human osteoblasts: novel mechanism of glucocorticoid-induced osteoporosis. *Biochem Biophys Res Commun* 2004;**318**:259–264.

52 H. D. Jang, J. H. Shin, D. R. Park, *et al*. Inactivation of glycogen synthase kinase-3β is required for osteoclast differentiation. *J Biol Chem* 2011;**286**:39043–39050.

53 J. Albers, J. Keller, A. Baranowsky, *et al*. Canonical Wnt signaling inhibits osteoclastogenesis independent of osteoprotegerin. *J Cell Biol* 2013;**200**:537–549.

54 W. Wei, D. Zeve, J. M. Suh, *et al*. Biphasic and dosage-dependent regulation of osteoclastogenesis by β-catenin. *Mol Cell Biol* 2011;**31**:4706–4719.

55 J. H. Kim and N. Kim. Regulation of NFATc1 in osteoclast differentiation. *J Bone Metab* 2014;**21**:233–241.

56 T. Watanabe, T. Kukita, A. Kukita, *et al*. Direct stimulation of osteoclastogenesis by MIP-1α: evidence obtained from studies using RAW264 cell clone highly responsive to RANKL. *J Endocrinol* 2004;**180**:193–201.

57 T. Kukita, N. Wada, A. Kukita, *et al*. RANKL-induced DC-STAMP is essential for osteoclastogenesis. *J Exp Med* 2004;**200**:941–946.

58 H. Takayanagi, S. Kim, T. Koga, *et al*. Induction and activation of the transcription factor NFATc1 (NFAT2) integrate RANKL signaling in terminal differentiation of osteoclasts. *Dev Cell* 2002;**3**:889–901.

59 J. R. Gillespie, V. Ulici, H. Dupuis, *et al*. Deletion of glycogen synthase kinase-3β in cartilage results in up-regulation of glycogen synthase kinase-3α protein expression. *Endocrinology* 2011;**152**: 1755–1766.

60 M. Grellier, N. Ferreira-Tojais, C. Bourget, *et al*. Role of vascular endothelial growth factor in the communication between human osteoprogenitors and endothelial cells. *J Cell Biochem* 2009;**106**: 390–398.

61 H. P. Gerber, T. H. Vu, A. M. Ryan, *et al*. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. *Nat Med* 1999;**5**:623–628.

62 H. Natsume, H. Tokuda, R. Matsushima-Nishiwaki, *et al*. Wnt3a upregulates transforming growth factor-β-stimulated VEGF synthesis in osteoblasts. *Cell Biochem Funct* 2011;**29**:371–377.

63 A. Kondo, H. Tokuda, J. Mizutani, *et al*. Wnt3a upregulates prostaglandin F2α-stimulated vascular endothelial growth factor synthesis in osteoblasts. *Mol Med Rep* 2012;**6**:421–425.

64 S. Kwan Tat, M. Padrines, S. Théoleyre, *et al*. IL-6, RANKL, TNF-α/IL-1: interrelations in bone resorption pathophysiology. *Cytokine Growth Factor Rev* 2004;**15**:49–60.

65 B. Sen, Z. Xie, N. Case, *et al*. Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable β-catenin signal. *Endocrinology* 2008;**149**:6065–6075.

66 B. Sen, M. Styner, Z. Xie, *et al*. Mechanical loading regulates NFATc1 and β-catenin signaling through a GSK3β control node. *J Biol Chem* 2009;**284**:34607–34617.

67 M. R. Steensma, W. K. Tyler, A. G. Shaber, *et al.* Targeting the giant cell tumor stromal cell: functional characterization and a novel therapeutic strategy. *PLoS ONE* 2013;**8**:e69101.

68 R. Hoessel, S. Leclerc, J. A. Endicott, *et al.* Indirubin, the active constituent of a Chinese antileukaemia medicine, inhibits cyclin-dependent kinases. *Nat Cell Biol* 1999;**1**:60–67.

69 S. Leclerc, M. Garnier, R. Hoessel, *et al.* Indirubins inhibit glycogen synthase kinase-3β and CDK5/p25, two protein kinases involved in abnormal tau phosphorylation in Alzheimer's disease. A property common to most cyclin-dependent kinase inhibitors? *J Biol Chem* 2001;**276**:251–260.

70 F. S. Wang, J. Y. Ko, L. H. Weng, *et al.* Inhibition of glycogen synthase kinase-3β attenuates glucocorticoid-induced bone loss. *Life Sci* 2009;**85**:685–692.

71 T. Watanabe, Y. Tamamura, A. Hoshino, *et al.* Increasing participation of sclerostin in postnatal bone development, revealed by three-dimensional immunofluorescence morphometry. *Bone* 2012;**51**:447–458.

72 U. Krause, S. Harris, A. Green, *et al. Pharmaceutical modulation of canonical Wnt signaling in multipotent stromal cells for improved osteoinductive therapy. Proc Natl Acad Sci U S A* 2010;**107**: 4147–4152.

73 J. Adachi, Y. Mori, S. Matsui, *et al.* Indirubin and indigo are potent aryl hydrocarbon receptor ligands present in human urine. *J Biol Chem* 2001;**276**:31475–31478.

74 N. H. Kulkarni, J. E. Onyia, Q. Zeng, *et al.* Orally bioavailable GSK-3α/β dual inhibitor increases markers of cellular differentiation *in vitro* and bone mass *in vivo*. *J Bone Miner Res* 2006;**21**:910–920.

75 N. H. Kulkarni, T. Wei, A. Kumar, *et al.* Changes in osteoblast, chondrocyte, and adipocyte lineages mediate the bone anabolic actions of PTH and small molecule GSK-3 inhibitor. *J Cell Biochem* 2007;**102**:1504–1018.

76 Q. L. Ying, J. Wray, J. Nichols, *et al.* The ground state of embryonic stem cell self-renewal. *Nature* 2008;**453**:519–523.

77 J. Bain, L. Plater, M. Elliott, *et al.* The selectivity of protein kinase inhibitors: a further update. *Biochem J* 2007;**408**:297–315.

78 L. A. Davis and N. I. Zur Nieden. Mesodermal fate decisions of a stem cell: the Wnt switch. *Cell Mol Life Sci* 2008;**65**: 2658–2674.

79 K. Kanke, H. Masaki, T. Saito, *et al.* Stepwise differentiation of pluripotent stem cells into osteoblasts using four small molecules under serum-free and feeder-free conditions. *Stem Cell Rep* 2014;**2**:751–760.

80 C. N. Bennett, K. A. Longo, W. S. Wright, *et al.* Regulation of osteoblastogenesis and bone mass by Wnt10b. *Proc Natl Acad Sci U S A* 2005;**102**:3324–3329.

81 J. E. Frith, D. M. Titmarsh, H. Padmanabhan, and J. J. Cooper-White. Microbioreactor array screening of Wnt modulators and microenvironmental factors in osteogenic differentiation of mesenchymal progenitor cells. *PLoS ONE* 2013;**8**:e82931.

82 K. Nakashima, X. Zhou, G. Kunkel, *et al.* The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. *Cell* 2002;**108**:17–29.

83 J. H. Hong, E. S. Hwang, M. T. McManus, *et al.* TAZ, a transcriptional modulator of mesenchymal stem cell differentiation. *Science* 2005;**309**:1074–1078.

84 L. Azzolin, F. Zanconato, S. Bresolin, *et al.* Role of TAZ as mediator of Wnt signaling. *Cell* 2012;**151**:1443–1456.

85 L. Azzolin, T. Panciera, S. Soligo, *et al.* YAP/TAZ incorporation in the β-catenin destruction complex orchestrates the Wnt response. *Cell* 2014;**158**:157–170.

86 R. Marsell, G. Sisask, Y. Nilsson, *et al.* GSK-3 inhibition by an orally active small molecule increases bone mass in rats. *Bone* 2012;**50**:619–627.

87 M. Eijken, I. M. Meijer, I. Westbroek, *et al.* Wnt signaling acts and is regulated in a human osteoblast differentiation dependent manner. *J Cell Biochem* 2008;**104**:568–579.

88 H. Clevers and R. Nusse. Wnt/β-catenin signaling and disease. *Cell* 2012;**149**:1192–1205.

89 F. Kugimiya, H. Kawaguchi, S. Ohba, *et al.* GSK-3β controls osteogenesis through regulating Runx2 activity. *PLoS ONE* 2007;**2**:e837.

90 M. Arioka, F. Takahashi-Yanaga, M. Sasaki, *et al.* Acceleration of bone development and regeneration through the Wnt/β-catenin signaling pathway in mice heterozygously deficient for GSK-3β. *Biochem Biophys Res Commun* 2013;**440**:677–682.

91 P. Clément-Lacroix, M. Ai, F. Morvan, *et al.* Lrp5-independent activation of Wnt signaling by lithium chloride increases bone formation and bone mass in mice. *Proc Natl Acad Sci U S A* 2005;**102**:17406–17411.

92 W. G. Gunn, U. Krause, N. Lee, and C. A. Gregory. Pharmaceutical inhibition of glycogen synthetase kinase-3β reduces multiple myeloma-induced bone disease in a novel murine plasmacytoma xenograft model. *Blood* 2011;**117**:1641–1651.

93 K. R. Georgiou, M. A. Scherer, C. M. Fan, *et al.* Methotrexate chemotherapy reduces osteogenesis but increases adipogenic potential in the bone marrow. *J Cell Physiol* 2012;**227**:909–918.

94 C. M. Edwards, J. R. Edwards, S. T. Lwin, *et al.* Increasing Wnt signaling in the bone marrow microenvironment inhibits the development of myeloma bone disease and reduces tumor burden in bone *in vivo*. *Blood* 2008;**111**:2833–2842.

95 K. Adamowicz, H. Wang, R. Jotwani, *et al.* Inhibition of GSK3 abolishes bacterial-induced periodontal bone loss in mice. *Mol Med* 2012;**18**:1190–1196.

96 Y. Tang, X. Zhou, B. Gao, *et al.* Modulation of Wnt/β-catenin signaling attenuates periapical bone lesions. *J Dent Res* 2014;**93**: 175–182.

97 Y. Chen, H. C. Whetstone, A. C. Lin, *et al.* Beta-catenin signaling plays a disparate role in different phases of fracture repair: implications for therapy to improve bone healing. *PLoS Med* 2007;**4**: e249.

98 G. Sisask, R. Marsell, A. Sundgren-Andersson, *et al.* Rats treated with AZD2858, a GSK3 inhibitor, heal fractures rapidly without endochondral bone formation. *Bone* 2013;**54**:126–132.

99 S. Sokol, J. L. Christian, R. T. Moon, and D. A. Melton. Injected Wnt RNA induces a complete body axis in *Xenopus* embryos. *Cell* 1991;**67**:741–752.

100 A. Suzuki, R. S. Thies, N. Yamaji, *et al.* A truncated bone morphogenetic protein receptor affects dorsal-ventral patterning in the early Xenopus embryo. *Proc Natl Acad Sci U S A* 1994;**91**: 10255–10259.

101 Y. Sasai, B. Lu, H. Steinbeisser, *et al. Xenopus* chordin: a novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 1994;**79**:779–790.

102 D. S. Kessler. Siamois is required for formation of Spemann's organizer. *Proc Natl Acad Sci U S A* 1997;**94**:13017–13022.

103 G. Carnac, L. Kodjabachian, J. B. Gurdon, and P. Lemaire. The homeobox gene *Siamois* is a target of the Wnt dorsalisation pathway and triggers organiser activity in the absence of mesoderm. *Development* 1996;**122**:3055–3065.

104 J. Li, N. Zhang, X. Huang, *et al*. Dexamethasone shifts bone marrow stromal cells from osteoblasts to adipocytes by C/EBPα promoter methylation. *Cell Death Dis* 2013;**4**:e832.

105 R. L. Jilka, R. S. Weinstein, K. Takahashi, *et al*. Linkage of decreased bone mass with impaired osteoblastogenesis in a murine model of accelerated senescence. *J Clin Invest* 1996;**97**:1732–1740.

106 K. R. Georgiou, T. J. King, M. A. Scherer, *et al*. Attenuated Wnt/β-catenin signalling mediates methotrexate chemotherapy-induced bone loss and marrow adiposity in rats. *Bone* 2012;**50**:1223–1233.

107 D. Silkstone, H. Hong, and B. A. Alman. Beta-catenin in the race to fracture repair: in it to Wnt. *Nat Clin Pract Rheumatol* 2008;**4**: 413–419.

108 B. G. Durie, B. Van Ness, C. Ramos, *et al*. Genetic polymorphisms of *EPHX1*, *Gsk3β, TNFSF8* and myeloma cell *DKK-1* expression linked to bone disease in myeloma. *Leukemia* 2009;**23**:1913–1919.

109 Y. Zhou, S. Uddin, T. Zimmerman, *et al*. Growth control of multiple myeloma cells through inhibition of glycogen synthase kinase-3. *Leuk Lymphoma* 2008;**49**:1945–1953.

110 F. Piazza, S. Manni, and G. Semenzato. Novel players in multiple myeloma pathogenesis: role of protein kinases CK2 and GSK3. *Leuk Res* 2013;**37**:221–227.

111 A. Zamani, G. R. Omrani, and M. M. Nasab. Lithium's effect on bone mineral density. *Bone* 2009;**44**:331–334.

112 P. Vestergaard, L. Rejnmark, and L. Mosekilde. Reduced relative risk of fractures among users of lithium. *Calcif Tissue Int* 2005;**77**:1–8.

113 I. Wilting, F. de Vries, B. M. Thio, *et al*. Lithium use and the risk of fractures. *Bone* 2007;**40**:1252–1258.

114 J. Frith and P. Genever. Transcriptional control of mesenchymal stem cell differentiation. *Transfus Med Hemother* 2008;**35**:216–227.

115 R. McBeath, D. M. Pirone, C. M. Nelson, *et al*. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004;**6**:483–495.

116 R. Torensma, H. J. Prins, E. Schrama, *et al*. The impact of cell source, culture methodology, culture location, and individual donors on gene expression profiles of bone marrow-derived and adipose-derived stromal cells. *Stem Cells Dev* 2013;**22**:1086–1096.

117 B. J. Jansen, C. Gilissen, H. Roelofs, *et al*. Functional differences between mesenchymal stem cell populations are reflected by their transcriptome. *Stem Cells Dev* 2010;**19**:481–490.

118 S. Maxson and K. J. Burg. Conditioned media cause increases in select osteogenic and adipogenic differentiation markers in mesenchymal stem cell cultures. *J Tissue Eng Regen Med* 2008;**2**:147–154.

119 A. P. Hall, K. J. Escott, H. Sanganee, and K. C. Hickling. Preclinical toxicity of AZD7969: effects of GSK3β inhibition in adult stem cells. *Toxicol Pathol* 2015;**43** (3):384–399.

CHAPTER 14

# Early molecular events during *in vitro* chondrogenesis

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## 14.1 Introduction

Cartilaginous tissues are found in many parts of the body, and as three different types: elastic, hyaline, and fibrocartilage. Elastic cartilage is found in the external ear, the auditory tube, and the epiglottis; fibrocartilage is found mainly in the intervertebral discs and in the menisci; and hyaline cartilage is found covering the articular surfaces of synovial joints. The functional difference between these tissues is determined by the composition of their extracellular matrix (ECM).

The ECM is a collection of extracellular molecules secreted by cells that provides structural and biochemical support to the surrounding cells. The composition of the ECM varies between multicellular structures. However, cell adhesion, cell-to-cell communication, and differentiation are common functions of the ECM. The ECM includes the interstitial matrix and the basement membrane. Gels of polysaccharides and fibrous proteins fill the interstitial space and act as a compression buffer against the stress placed on the ECM. Basement membranes are sheet-like depositions of ECM on which various epithelial cells rest.

As the vast majority of *in vitro* cartilage research is focused on hyaline cartilage, this will be the tissue of interest for this chapter.

Research on *in vitro* hyaline cartilage formation may be performed in order to establish a model system for cartilage diseases, or as a model to study the *in vitro* differentiation of mesenchymal stromal cells (MSCs) to the end-differentiated

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articular chondrocytes (ACs), but mostly this research is motivated by the desire to create tissue-engineered cartilage for repair of lesions of articular cartilage. This is a highly desirable endpoint because lesions of articular cartilage do not usually heal spontaneously. A persisting lesion with increasing pain and reduced functionality of the joint usually leads to surgical intervention. Today, the most frequently used surgical technique is microfracture of the bone plate between the cartilage lesion and the bone marrow. This leads to the release of bone marrow and blood into the lesion cavity with the subsequent formation of a clot. Over time the clot will be replaced by fibrocartilage [1]. This will provide symptomatic relief initially, but the long-term result is uncertain, with osteoarthritis (OA) a relatively common outcome. The modern alternative to microfracture surgery is to implant cells in order to regenerate the lost cartilage. This strategy was initiated by Brittberg *et al.* in 1994 when they removed a small piece of cartilage from a damaged knee through an arthroscope, isolated single chondrocytes from the biopsy, expanded the cells *in vitro*, and implanted them back into the lesion covered by a flap [2]. This procedure, autologous chondrocyte implantation (ACI), has been followed by second-generation procedures in which synthetic membranes, able to act as cell carriers, have replaced the flap. In third-generation procedures the ACs are established in a biomaterial before implantation. However, a 2010 Cochrane review concluded that there was no evidence that ACI of any generation performed better than microfracture surgery [3]. This may be because ACs, when they are expanded in monolayer cultures *in vitro*, lose their ability to produce ECM molecules typical of hyaline cartilage, such as type II collagen (COL2) and aggrecan, and begin instead to produce COL1 and versican in a process called dedifferentiation [4].

When the cartilage biopsy is taken in order to get tissue for isolation of ACs, a small injury to the healthy cartilage is created. Although this lesion is frequently situated in the periphery of the articular cartilage, outside that part of the cartilage which functionally interacts with the cartilage covering the end of the opposing bone, there is still a risk of morbidity to this procedure [5]. To avoid this problem, MSCs may be used instead. MSCs can be obtained from several sites, particularly from the bone marrow, with minimal pain and no morbidity. Human adult MSCs may resemble the mesenchymal cells from which cartilage and bone are formed during embryogenesis [6]. If so, it should be possible to mimic the developmental stages involved in embryological chondrogenesis when MSCs are differentiated to chondrocytes *in vitro*. Developmental chondrogenesis has been carefully studied and well reviewed, based mostly on studies in rodents [7–9] and humans with developmental disorders of cartilage and bone [6]. Recently, data were published based on studies of human fetal tissues [10]. The purpose of this chapter is to review current knowledge about the early molecular changes observed during *in vitro* chondrogenesis and then, where possible, to compare this with *in vivo* chondrogenesis. To do so we need to first review the composition of adult articular cartilage and its developmental history.

## 14.2 Adult articular cartilage

Where mobile bones meet, the areas of contact are covered by a highly specialized tissue called hyaline cartilage. Because this hyaline cartilage is part of the joint, it is also frequently called articular cartilage. Articular cartilage has no blood supply and no nerve supply, which are probably the main reasons why lesions of this tissue heal poorly or not at all. The cells make up only 2–3% of the volume of the cartilage, the rest being ECM and water. All the cells are end-differentiated chondrocytes, but their ECM synthesis profile varies a little depending on their anatomical situation within the cartilage.

The most abundant ECM protein is COL2. These are homotrimeric molecules, synthesized as procollagens with propeptides at both ends that need to be cleaved off after secretion from the chondrocytes in order for fibrillation to occur. COL2 fibrils also contain small amounts of COL9, localized at the fibril surfaces, and COL11, which is important for regulation of fibril diameter. The collagen fibrils provide tensile strength to the cartilage. Other collagens in articular cartilage include COL6 (found predominantly in the pericellular region), COL1 (synthesized by some of the chondrocytes near the cartilage surface), and COL10 (synthesized sparsely in the calcified zone of articular cartilage near the subchondral bone).

The major proteoglycan in articular cartilage is aggrecan. Aggrecan is modified by side chains of glycosaminoglycans (GAGs), such as chondroitin and keratan sulfate and other oligosaccharides, and forms large aggregates with the long molecules of hyaluronic acid linked together by the link protein. These aggregates bind water and give cartilage its compressive strength. Proteoglycan 4, also called lubricin, is found on the surface of articular cartilage. It serves to reduce friction between the two articular surfaces [11].

Other molecules of importance are cartilage oligomeric protein (COMP) of the thrombospondin family, fibronectin, matrilins, fibrilins, and chondroadherins. The functional roles played by these molecules are incompletely understood.

## 14.3 Developmental chondrogenesis

The limb skeleton is formed from lateral plate mesoderm cells that migrate to the sites where the skeletal elements will develop. Here, they form mesenchymal condensations of high cell density [12]. These cells proliferate and begin to differentiate into chondroprogenitor cells. Further differentiation leads to the formation of chondrocytes. These cells continue to proliferate, but they also start to produce ECM, which, with the chondrocytes, makes up cylindrical structures called cartilage anlagen, produced in sequence from proximal to distal ends [6]. Beginning in the center of the anlagen, the chondrocytes subsequently exit the cell cycle and increase their cell volume to become hypertrophic chondrocytes. Through secretion of angiogenic factors, these cells induce sprouting angiogenesis from the

surrounding perichondrium. The vessels allow invasion of osteoblasts, osteoclasts, and hematopoietic cells; the hypertrophic chondrocytes undergo apoptosis, and cartilage is replaced by bone in what is called the primary ossification center [6,7,9]. At the same time, osteoblasts in the perichondrium around the middle portion of the cartilage anlagen, called the diaphysis, form a bony collar around the anlagen [6]. The rounded ends of the anlagen are called the epiphysis. Between the diaphysis and the epiphysis, in a region called the metaphysis, secondary ossification centers are formed at both ends of the anlagen. Here, a plate of cartilage called the growth plate becomes the site for bone elongation through a coordinated sequence of chondrocyte proliferation, hypertrophy, apoptosis, and replacement by osteoblasts and osteoclasts and subsequent bone formation [13].

Starting from the prechondrogenic condensations, there is now evidence for the development of two different types of chondrocytes. One is the type just described, which gives rise to the cartilage anlagen and becomes replaced by bone in the process called endochondral ossification. The other type of chondrocyte is formed from mesenchymal cells developing in a region called the interzone that forms at the sites of prospective joints. Some of the cells in the interzone die by apoptosis, and the space left behind becomes the synovial cavity. Cells in the interzone layer closest to the epiphyseal part of the anlagen differentiate to become ACs which produce the hyaline ECM of articular cartilage [14,15].

## 14.4 Molecular aspects of *in vivo* chondrogenesis

The molecular events associated with the developmental features described above are complex and still incompletely understood. In this Chapter only those aspects of gene expression and protein synthesis that are most relevant for *in vitro* chondrogenesis will be described. It should be noted, however, that most of what is known today about molecular aspects of *in vivo* chondrogenesis stems from chondrogenesis occurring within the cartilage anlagen. Scientists attempting to make cartilage from MSCs *in vitro* may perhaps be better served by trying to imitate the process by which cells of the mesenchymal condensations differentiate through interzone cells to become ACs. Unfortunately, this process is less well described.

In the mesenchymal condensations, important cell adhesion molecules are neural cadherin (N-cadherin) and neural cell adhesion molecule. The ECM is composed mainly of fibronectin, COL1, the IIA splice variant of COL2, and COMP (Figure 14.1) [7].

The transcription factor (TF) most closely associated with *in vivo* chondrogenesis is Sry-like homeobox TF (SOX) 9. *SOX9* transcripts are detected in prechondrogenic mesenchymal condensations as early as 8.5–9.5 days of mouse embryonic development. However, the stage of mesenchymal condensation is thought to last until day 11.5 to 12.5, at which time *SOX6* and some other *SOX* genes are also expressed [8]. At early stages of mesenchymal condensation patterning, systems such as fibroblast growth factors (FGFs), hedgehog, bone morphogenetic proteins (BMPs), and wingless-type mouse mammary tumor virus integration site family (WNTs) coordinate signaling along the three axes of the limb [7]. However, perhaps the most important signaling axis at this stage occurs between growth differentiation factor 5 (GDF5) and its receptor BMP receptor 1B (BMPR1B) [10,16]. Expression of *GDF5* was found to differentiate cells within the mesenchymal condensations toward interzone cells. These cells, in turn, gave rise to articular cartilage, the synovial lining, and other joint tissues, but

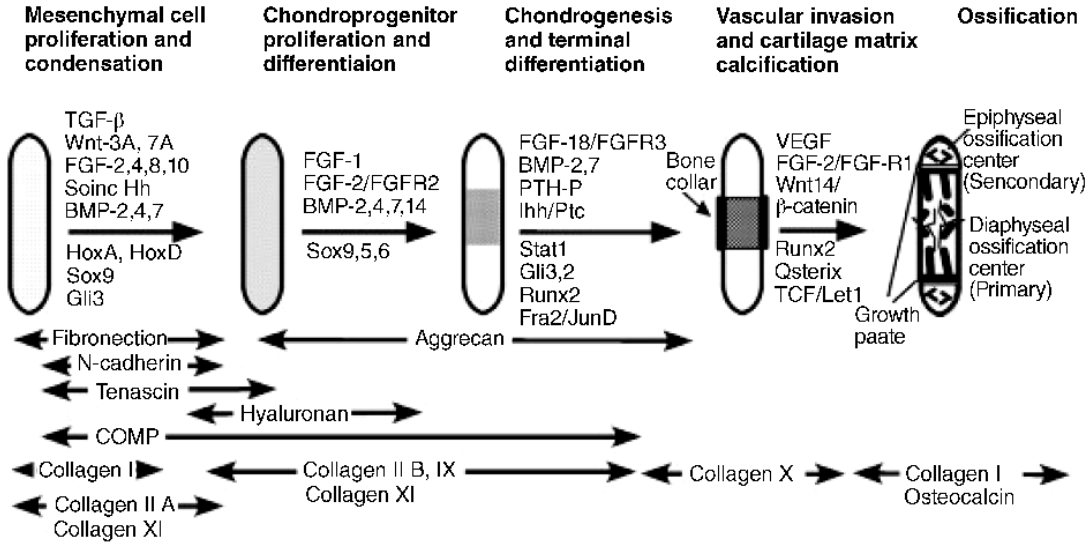



**Figure 14.1** Sequence of events of chondrogenesis during the development of long bones. The different stages are represented schematically, showing the temporal patterns of growth and differentiation factors above the arrows and the TFs involved below the arrows. The ECM proteins that distinguish the different stages are indicated below. *Source*: [7] Goldring, M.B. *et al.*, *J Cell Biochem* 2006; **97**, 33–44.

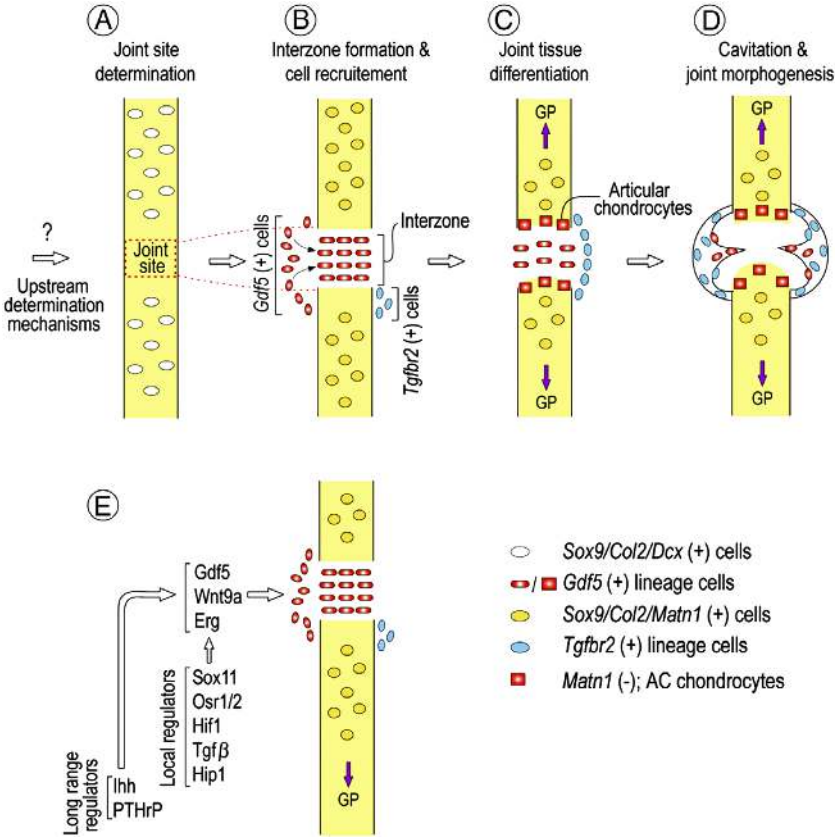



**Figure 14.2** Generalized model of limb joint formation and morphogenesis. (A) At early developmental stages, an as-yet unknown upstream determination mechanism would identify and prescribe the location of the joints along *SOX9*/*COL2*/*DCX*-expressing anlagen. (B) Soon after, *GDF5* expression would be activated along with other interzone-specific genes (see (E)) that would define the initial interzone mesenchymal population within the *SOX9*/*COL2*/*MATN1*-positive cartilaginous anlagen. This would be accompanied by cell immigration from the flank, and cells located dorsally and ventrally would activate *TGFBR2* expression. (C) *GDF5*-positive cells adjacent to their respective cartilaginous anlagen, with a *SOX9*/*COL2* history but negative for *matrilin-1* expression, would differentiate into ACs. (D) Additional differentiation processes and mechanisms, such as muscle movement, would bring about cavitation and genesis of other joint tissues, such as ligaments and other meniscus tissue, involving *GDF5*- and *TGFBR2*-positive and -negative cell progenies. (E) Schematic summarizing local and long-range regulators that converge to regulate interzone gene expression at the early stages of joint formation. *Source*: [16] Decker 2014, pp. 5–10. Reproduced with permission of Elsevier.

contributed little to the development of the cartilage anlagen. *GDF5* null mutants formed functionally defective joints [15]. The development of articular cartilage from interzone cells requires the hypoxia regulator HIF-1α to function, and SOX11, TGF-β, WNT9a, and odd-skipped related 1/2 (OSR1/2) were also found to be involved in this process (Figure 14.2) [16]. Additionally, during human fetal development, *GDF5* and its *BMPR1B* receptor were found to be highly expressed in prechondrocytes, with much higher expression in prechondrocytes than in the cartilage anlagen [10].

Through the stage of chondroprogenitor proliferation and the formation of cartilage anlagen, *SOX9* and *SOX6* remain highly expressed; *SOX5*, the last member of the so-called Sox trio, is barely expressed, while *SOX8* is considerably upregulated. Several forkhead/winged helix box (*FOX*) and *BMP* genes are upregulated, while TFs of the T-box (*TBX*) and homeobox (*HOX*) families are downregulated [8]. Col2 now switches to the IIB splice variant, and transcripts for other ECM molecules, such as aggrecan (*Acan*), *Comp*, and the link protein hyaluronan and proteoglycan link protein 1 (*Hapln1*), are greatly upregulated. This is also the case for matrilin 1, which has been shown not to be expressed in the interzone, but rather in the anlagen chondrocytes [8,17].

As the cells change from terminally differentiated to hypertrophic chondrocytes, a series of fundamental changes occurs in the repertoire of molecules produced by the cells. The SOX trio TFs cease to be produced and, as a consequence, Col2, Col9, Col11, and aggrecan are also no longer secreted by the cells. Instead, runt related TF 2 (*Runx2*), the TF most essentially involved in bone formation, is produced; vascular endothelial growth factor production leads to ingrowth of blood vessels, and the new blood supply brings with it osteoblasts, osteoclasts, and matrix metalloproteinases (MMPs), produced by the hypertrophic chondrocytes. At this stage, *COL10* is greatly upregulated and serves as a marker for chondrocyte hypertrophy. As hypertrophic chondrocytes die by apoptosis they are replaced by osteoblasts, which lay down new ECM typical of bone, and this ECM is subsequently calcified under the influence of alkaline phosphatase [6,7]. These changes begin to occur at the primary ossification center and then spread along the shaft (diaphysis) of the anlagen, which gradually becomes a bony tube. The process of proliferating chondrocytes changing to become hypertrophic chondrocytes that die and become replaced by osteoblasts continues to lead to elongation of the bone shaft in the secondary ossification center until the skeleton is fully developed. At this stage the remaining cartilage is replaced by bone, and the only cartilage left is the hyaline cartilage covering the articular surface at the end of the bone.

## 14.5 Determinants of *in vitro* chondrogenesis

The molecular events occurring during *in vitro* chondrogenesis may vary depending on the cells used, the culture medium, the differentiation cocktail, and the three-dimensional (3D) culture used for the chondrogenic differentiation of the MSCs. These determinants of *in vitro* chondrogenesis also need to be discussed before the early molecular events during *in vitro* chondrogenesis are described.

## 14.6 Tissue source of mesenchymal stromal cells

MSCs are found in almost all tissues in the body, but the tissues used most to provide MSCs for *in vitro* chondrogenesis are the bone marrow MSCs (BM-MSCs), adipose tissue MSCs (AT-MSCs), and synovial membrane MSCs (SY-MSCs). Most comparative studies have been performed between BM-MSCs and AT-MSCs [18]. Of relevance, adipose tissue yields a much higher number of MSCs per weight of tissue, and the proliferative capacity of AT-MSCs is higher. However, most studies conclude that BM-MSCs have a better chondrogenic differentiation capability than AT-MSCs [19–22]. SY-MSCs are relatively easily accessible, and have been compared with other sources of MSCs [23]: SY-MSCs were found to have a higher proliferative capacity and showed better *in vitro* chondrogenic differentiation capability than MSCs from any other source. Thus, although MSCs from all tissues by definition should differentiate to become chondrocyte-like cells, the best choices for *in vitro* chondrogenesis seem to be BM-MSCs and SY-MSCs.

Recently, the use of co-cultures of MSCs and ACs has been investigated [24]. This is predominantly done to reduce dedifferentiation and enhance redifferentiation of the chondrocytes, but the addition of chondrocytes to enhance chondrogenic differentiation and prevent hypertrophy of the differentiated MSCs has also been investigated. Although the results vary somewhat, the general impression from these studies is that co-culture with chondrocytes improves the expression of chondrogenic genes, enhances the synthesis of cartilaginous ECM, and reduces the expression of marker genes for hypertrophy. The mechanism for the latter effect may be through the secretion of parathyroid hormone related protein by chondrocytes [24].

### 14.6.1 *In vitro* cell culture

In order to obtain the best results from assays of *in vitro* chondrogenesis, the materials and reagents used for the expansion cell cultures are important. From the early days of MSC research it has been known that MSCs require fetal bovine serum (FBS) for rapid proliferation to occur, and that there are considerable differences between different batches of FBS in their ability to support MSC proliferation [25]. Additionally, the type of culture flask used has been shown to impact on MSC proliferative capacity [26]. There may also be differences between culture media in their ability to support cell proliferation versus chondrogenic differentiation [27].

If *in vitro* chondrogenic differentiation of MSCs is performed with the aim of providing tissue for repair of lesions of human articular cartilage, the use of FBS may be unfortunate because it carries the risk of inducing xenogeneic immune responses and it may contain zoonotic agents or prions [28–30]. Regulatory agencies in several countries will not accept the use of MSCs cultured in FBS as human medicinal products [31]. One alternative may be the use of autologous serum, which is easily available, free of charge, and acceptable to all regulatory authorities. Using BM-MSCs and serum from healthy donors we showed that MSCs cultured in autologous serum proliferated faster and showed better *in vitro* chondrogenic differentiation capability than if FBS was used [32]. However, MSCs from patients with advanced OA have reduced *in vitro* proliferation capacity, and it is our unpublished impression that serum from these patients occasionally may also be less supportive of *in vitro* proliferation than serum from healthy donors (Brinchmann JE, unpublished observations) [33]. The mechanisms behind these observations have not yet been determined. However, the reduced proliferation rate may be counterbalanced by the addition of FGF2 [34,35]. Using allogeneic human serum alone is not a good alternative: in most cases MSCs will not expand adequately *in vitro* under such circumstances [32,36]. However, if allogeneic human AB plasma is spiked with human platelet lysate, the proliferation rate will usually be found to supersede that observed with FBS, and *in vitro* chondrogenesis is as good or better [37,38]. This serum supplement is likely to be acceptable to regulatory authorities, but it should be kept in mind that platelet lysate contains a large number of growth and differentiation factors, the impact of each of which has not yet been fully determined.

In addition to the right choice of plastic, medium, serum, and growth factors, the chondrogenic differentiation of MSCs may also be affected by the concentration of oxygen in the incubator atmosphere. Normally, the concentration of oxygen in the incubator atmosphere is around 20%. With the cells in monolayer culture covered by only a thin layer of medium, oxygen consumed by the cells is rapidly replaced by oxygen from the incubator air. This supports aerobic respiration in the proliferating MSCs. This makes the milieu surrounding the proliferating MSCs very different from that of the mesenchymal condensations and their chondrogenically differentiating offspring. This tissue is avascular and hypoxic and entirely dependent on hypoxia-inducible factor-1 (HIF-1) [39]. Following conditional knock-out of *HIF-1α*, Provot *et al.* observed malformation of the limbs and joints [40]. However, the impact observed by culturing MSCs in oxygen-depleted atmosphere *in vitro* varies between species and also varies between different studies [41]. Most impressive, perhaps, is the effect observed by differentiating micromasses of human MSCs in either room air or hypoxia (2.5%). Here, differentiation into articular, permanent-type chondrocytes, and

inhibition of genes associated with hypertrophy was observed under hypoxic conditions [42]. However, for these experiments the MSCs were first expanded *in vitro* in monolayer with room air in the incubator atmosphere.

## 14.7 Three-dimensional culture systems and bioscaffolds

Optimal chondrogenic differentiation of MSCs *in vitro* requires some sort of 3D culture system. In part this has to do with the organization of the cytoskeleton and focal adhesions: *in vitro* chondrogenesis could be shown to be encouraged by the cells having a rounded shape without focal adhesion attachments [43]. Classically, this has been achieved by gently centrifuging the cells to form a pelleted micromass, perhaps resembling the mesenchymal condensations of embryological chondrogenesis [44].

Micromass culture systems have been extensively used to characterize aspects of *in vitro* chondrogenesis, but until recently this model system has not been applied to strategies of *in vitro* chondrogenesis for clinical use. Using a model system where *in vitro* expanded human MSCs were cultured for a specified period in aggregates called condensed mesenchymal bodies in medium supplemented with TGF-β3 and dexamethasone, Bhumiratana *et al.* tried to mimic embryological chondrogenesis [45]. They then pressed a layer of the condensed mesenchymal bodies on to porous decellularized bone matrix. After 5 weeks of cultivation, hyaline cartilage with clinically relevant thickness, width, and compression capability was formed. This model is perhaps too cumbersome for routine production of autologous cartilage or osteochondral implants, but may arguably be the best and most physiologically appropriate model system for *in vitro* chondrogenesis published to date.

Most groups working toward clinically applicable systems for cartilage tissue engineering use biomaterial scaffolds in their efforts [41]. The scaffolds may be made from naturally occurring proteins, polysaccharides, or polyesters, or from various types of synthetic biomaterials. The biomaterials may have different formats, such as gels, foams, sponges, or membranes. The best biomaterial has not yet been identified, but the winner should be biocompatible and most likely biodegradable. It may also turn out to be an advantage if the biomaterial is able to contain growth or differentiation factors. Data on early molecular events during *in vitro* chondrogenesis derive from many of these model systems and may help to distinguish useful combinations of cells, biomaterials, and growth or differentiation factors.

## 14.8 Epigenetic changes during early *in vitro* chondrogenesis

### 14.8.1 An introduction to epigenetics

Epigenetics (see Chapter 6) is the name used to describe a heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence. Briefly, the basic unit of chromatin is the nucleosome. The nucleosome consists of homodimers of each of the four histones H2A, H2B, H3, and H4, forming a histone octamer (Figure 14.3A). DNA encircles the

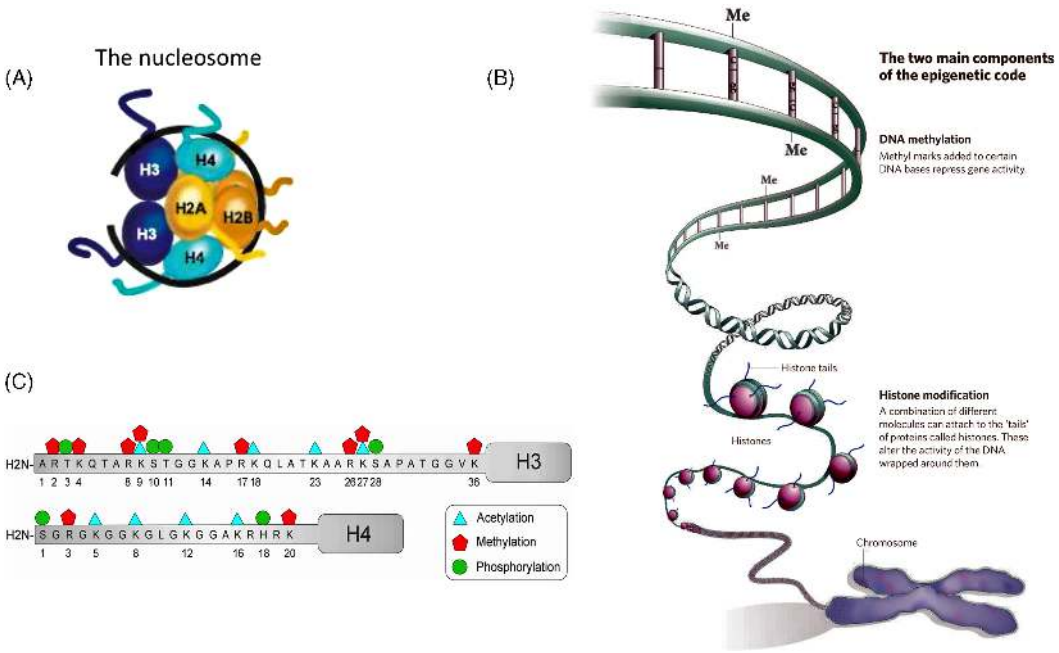



**Figure 14.3** Schematic presentation of components of the epigenetic code. (A) Cross-section of the nucleosome. (B) Diagram describing both components: methylation at CpG dinucleotides and histone modifications. (C) Some of the most common modifications of the tails of histones H3 and H4. *Sources*: Philippe Collas (A and C) and [46] Qiu J, *Nature* 2006; **441**: 143.

histone octamer nearly twice, before moving on to encircle the next histone octamer. The DNA that is not involved in the nucleosome is associated with the linker histone H1. The nucleosomal histones all have tails that protrude from the nucleosome. Chromatin is organized into higher order structures, including loops and folds; there is variation in the density of chromatin packing, and the position of chromatin relative to the nuclear membrane is also considered an epigenetic parameter.

All the aspects of chromatin modification and organization described may impact on the level of gene expression. First, DNA nucleotides may be modified, most commonly by methylation of cytosine–phosphate–guanosine (CpG) dinucleotides (Figure 14.3B). CpG islands is the name given to short sequences of DNA with a higher than average proportion of CpG dinucleotides. These are commonly found in or near gene promoters. The degree of methylation of these CpG islands may impact strongly on gene expression.

Second, the protruding histone tails may be modified, most commonly the tails of histones H3 and H4. These tails may undergo any of a number of modifications involving many of the amino acids making up the tails (Figure 14.3C). The histone modifications have different types of impact on gene expression depending on the nature of the modification and the amino acid involved. The histone modifications that will be described here are all located in the tail of H3. Trimethylation of lysine at amino acid position number 4 (H3K4me3) is a promoter mark associated with transcriptional activation. The same goes for acetylation of lysine at position 9 (H3K9ac), also most commonly a promoter mark associated with gene expression. Another promoter mark, H3K27me3, is associated with silencing of genes. The mark H3K36me3, when it occurs, is commonly found throughout the gene body, and is closely associated with gene transcription.

Third, we will discuss two histone modifications that have been found to define active enhancers: H3K4me1 and H3K27ac. Higher order organization of the chromatin, such as loops and folds, may bring distally placed regulatory sequences into contact with promoter sequences or other regulatory factors near the transcription start site. When chromatin is packed loosely it is called euchromatin, while densely packed chromatin is called heterochromatin. Euchromatin allows access of the transcriptional machinery to DNA, and is associated with gene expression. Heterochromatin is more often found close to the nuclear membrane and is associated with gene silencing [47].

### 14.8.2 DNA methylation of the *COL2A1* and *COL10A1* promoters

Little has been published on epigenetic changes during *in vitro* chondrogenesis. In 2008, Richter's group investigated the methylation status of CpG islands in the *COL2A1* and *COL10A1* promoters in MSCs before and after chondrogenic differentiation of MSCs and in ACs in the uncultured, *in vitro* expanded and dedifferentiated state and after redifferentiation [48]. They found that CpG islands were unmethylated around the *COL2A1* transcription start site of all cell groups, independent of *COL2A1* expression. The situation was different for the *COL10A1* promoter: all nine CpG sites in the sparse *COL10A1* promoter in uncultured ACs were consistently methylated, although a low level of *COL10A1* mRNA was expressed. The same observation was made after chondrogenic redifferentiation of dedifferentiated ACs. Undifferentiated MSCs showed a state of partial methylation, with no expression of *COL10A1* mRNA. Upon differentiation, two of the partially methylated CpG sites became less methylated, and *COL10A1* mRNA increased dramatically. These observations are consistent with later observations made by our own group [49]. We found that, after chondrogenic differentiation of both cell types, differentiated MSCs expressed *COL10A1* mRNA at levels almost 100-fold higher than in redifferentiated ACs. When the cells in an alginate biomaterial differentiation scaffold were stained for COL10 protein, this was found in or around practically every differentiated MSC, but only in about 1% of the redifferentiated chondrocytes. We interpret this to mean that every MSC, when exposed to chondrogenic differentiation medium, will express *COL10A1* mRNA and protein, suggesting that the state of partial methylation of CpG islands in the undifferentiated MSCs does not represent an epigenetic block for *COL10A1* expression. For ACs, *COL10A1* expression is epigenetically blocked in practically all the cells, even after redifferentiation with a differentiation cocktail. The exception is approximately 1% of the cells, too few to impact on assays of CpG methylation of the whole population of chondrocytes and perhaps representing the cells in the calcified zone of articular cartilage known to express COL10 *in vivo*. For *COL2A1*, the lack of expression of mRNA in the undifferentiated MSCs must be independent of CpG methylation, as the islands near the promoter were all unmethylated.

### 14.8.3 DNA methylation of promoters in other chondrogenesis candidate genes

In terminally differentiated mammalian cells, approximately 60–80% of all CpGs are methylated [50]. Most CpGs in mammalian genomes remain methylated during development, but CpG islands found at promoters of many housekeeping and developmentally regulated genes are constitutively hypomethylated. As pluripotent stem cells differentiate toward specific lineages, genes associated with other lineages tend to become silenced, and this is associated with increased DNA methylation of their promoters. However, the exact relationship between differentiated phenotypes and DNA methylation status remains complex [50]. It was interesting, therefore, when Ezura *et al.* found that the promoters of 10 genes relevant to *in vitro* chondrogenesis were hypomethylated in human MSCs collected from joint synovium, both during *in vitro* expansion and after chondrogenic differentiation, and regardless of expression level of the genes [51]. One possible interpretation of these observations is that the synovium-derived MSCs were sufficiently close to the chondrocyte progenitors in developmental terms for genes of

importance to *in vivo* chondrogenesis to still be epigenetically accessible. Some of these genes were not expressed in the undifferentiated MSCs, like *COL2A1* in the study from Richter's group, suggesting that DNA methylation is not an essential aspect of gene regulation in these cells.

### 14.8.4 Genome-wide map of quantified epigenetic changes during *in vitro* chondrogenesis of bone marrow mesenchymal stromal cells

To the best of our knowledge, the only other study of epigenetic changes during *in vitro* chondrogenic differentiation of MSCs is our own work on genome-wide quantified changes in histone modifications and DNA methylation during *in vitro* chondrogenic differentiation of BM-MSCs [52]. Using cells from four donors, we performed assays on MSCs in monolayer culture on day 0 and after 7 days of chondrogenic differentiation in an alginate scaffold. At this time, all the changes in gene expression that will occur during *in vitro* chondrogenesis have begun. We identified a set of 10 genes that were important for chondrocyte functionality, and another set of 10 genes described to be important for MSC functionality, and showed how the expression of all the chondrocyte signature genes were upregulated and all the MSC signature genes were downregulated after 7 days of differentiation. Genes that were not described to have a role in chondrogenesis showed unchanged expression. We found that all the chondrogenesis signature genes had increased levels of H3K4me3, H3K9ac, and H3K36me3 and reduced levels of H3K27me3, while all the MSC signature genes had reduced levels of H3K4me3, H3K9ac, and H3K36me3 and increased levels of H3K27me3, showing an absolute correlation between changes in gene expression and changes in histone modifications for these signature clusters. In fact, there was a significant correlation between the magnitude of upregulation of H3K4me3 and the magnitude of increase in gene expression. No changes in histone modifications were observed for the control genes. Examples of ChIP-seq signal tracks for these histone modifications in MSCs and chondrogenically differentiated MSCs (Chondro) for the MSC gene *CXCL12* (also known as *SDF-1*) and for the chondrocyte gene *COL2A1* are shown in Figure 14.4. Note in the figure that, although *COL2A1* mRNA is expressed at relatively high level by day 7, H3K27me3 is reduced but still present in chondrogenically differentiated cells. Although not formally tested, we have taken this to suggest that the MSCs have not been differentiated in synchrony, so that the expression of *COL2A2* mRNA is still inhibited by H3K27me3 in some cells, while being expressed, presumably in the absence of H3K27me3 and the presence of H3K4me3, H3K9ac, and H3K36me3 in other cells.

Interestingly, we also found moderate upregulation of H3K4me3 and H3K9ac in a large number of genes with no significant increase in gene expression. Ontogenetically, these genes were linked to the ECM and the skeletal system. However, increased H3K36me3 was usually not found to be increased along the sequence of these genes. A similar phenomenon has also been observed in other model systems and has been called prepatterning of the genes [53]. We interpret this to mean that our differentiation cocktail, combined with placing the cells in the alginate scaffold, opened up an epigenetic landscape that could potentially allow differentiation into bone and other skeletal components. Then, under the influence of TFs and other elements, the actual transcription is restricted predominantly to those genes required for chondrogenesis. Upregulation of H3K36me3 was the single epigenetic mark most closely correlated with an increase in transcriptional activity.

The data obtained in this study also provide more information about the epigenetic regulation of *COL10A1* expression. Within the limitations given by the number of histone modifications examined, no histone marks at all were detected near the *COL10A1* transcription start site. After the onset of differentiation, the usual combination of histone marks were observed, including marks for local enhancer regions. Combined with the data described earlier, this suggests that epigenetic restriction to *COL10A1* transcription may indeed be provided by methylation of CpG islands in the promoter region, as the histone silencing mark H3K27me3 was not present near this gene in undifferentiated MSCs.

A genome-wide approach like the one used here can also be used to identify distant regulatory sequences. The two histone modifications used to identify enhancer regions in our study were H3K4me1 and H3K27ac. Upregulation of these marks co-occurred with high frequency. When we examined regions around *COL2A1*, we found six regions where these marks were increased (Figure 14.5A). Two of these had already been validated as *COL2A1* enhancers; the other four were new enhancer regions. However, to be functionally active enhancer regions one would expect them to contain binding sites for TFs known to be relevant for chondrogenesis. In many cases the sequences most likely to bind TFs are known. Using known binding motifs to scan the enhancer regions found near *COL2A1*, we found possible binding motifs for a number of TFs relevant for chondrogenesis in all the enhancer regions, including SOX9 and SP1/SP3 (Figure 14.5B) [41].

In this study, we also performed a genome-wide analysis of DNA methylation using a reduced representation bisulfite sequencing strategy. We found a highly significant correlation between the undifferentiated and chondrogenically differentiated MSCs ($r^2 = 0.78$), with the methylation states of the vast majority of promoters remaining unchanged. For the MSC and chondrocyte signature genes, promoters were hypomethylated both before and after differentiation, consistent with the observations made by Ezura *et al.*, although only *SOX9* and *CXCL12* were overlapping between their 10 candidate genes and our 20 signature genes [51]. This confirms the impression that DNA methylation is unlikely to play a major role in the regulation of

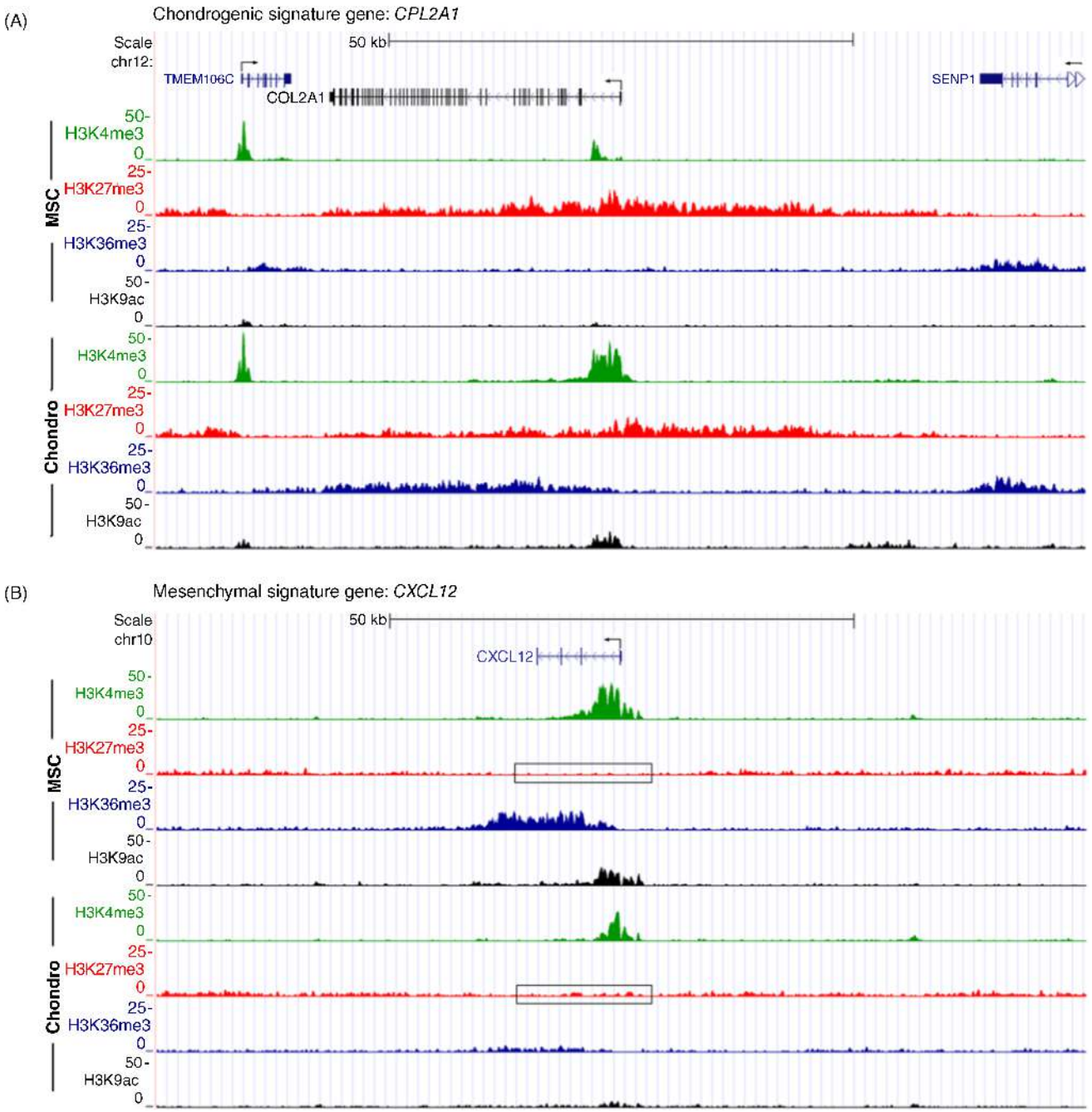


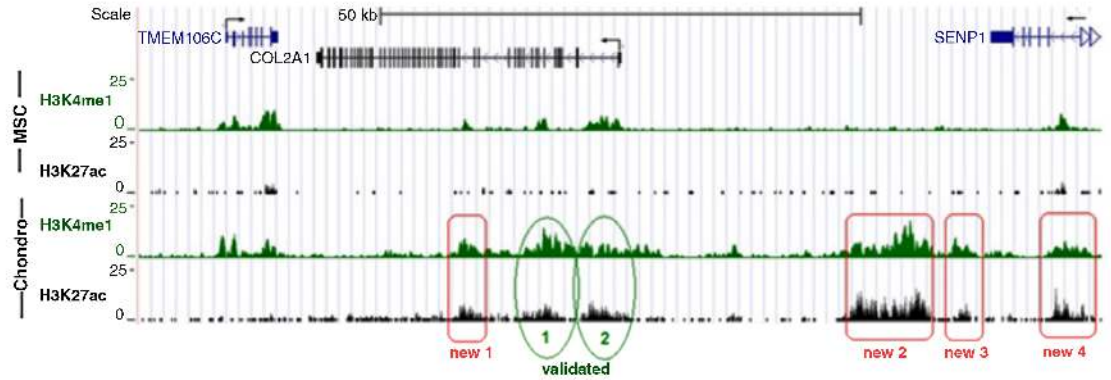

**Figure 14.4** (A) ChIP-seq signal tracks for histone modifications in a 100 kb window around the transcription start site of the chondrogenic marker gene *COL2A1*. Exon–intron structures and coding strand direction are depicted on top. (B) ChIP-seq signal tracks for histone modifications in a 100 kb window around the transcription start site of the MSC marker gene *CXCL12*. (See color plate section.) *Source*: [52] Herlofsen, S.R. *et al.*, *BMC Genomics* 2013; **14**, 105.

key chondrogenesis genes. Of note, *COL10A1* was not included in any of these series of genes, and the resolution of our method used for detecting DNA methylation was not sufficient to pick up the changes in DNA methylation reported in the study from Richter's group [48].

### 14.8.5 Epigenetics: conclusions

For the vast majority of genes of relevance to *in vitro* chondrogenesis of human MSCs the promoters are hypomethylated both before and after differentiation. This suggests that DNA methylation does not regulate the differentiation process in

(A) *COL2A1* regulatory areas




(B) Motif scanning in chondrocyte enriched K27ac areas

| Enriched TF-family | Member involved in chondrogenesis | Example motif (p-value, Fisher test) |
|---|---|---|
| SRY | SOX5, 9 | SOX9, (9.76E-03) |
| FOX | FOXA2+3, FOXCI+2, FOXE1 | FOXA2 (8.29E-06) |
| EBOX | ARNT, HIF1A, USF1 | ARNT (1.85E-04) |
| SP/KLF | SP1+3, MZF-1 | SP1(7.31E-05) |
| ETS | ETS-1, NFATC2 | EST-1 (6.43E-03) |
| HOMEOBOX | NKX3.2, AP1, HOXA5, PAX | AP1 (1.43E-02) |

Enriched motifs in *COL2A1* regulatory areas

| Regulatory area | TF-family - Motif hits |
|---|---|
| New 1 | SRY, EBOX, SP1/KLF, ETS, HOMEOBOX |
| Validated 1 | SRY, FOX, SP1/KLF, ETS, HOMEOBOX |
| Validated 2 | SRY, FOX, EBOX, SP1/KLF, ETS, HOMEOBOX |
| New 2 | SRY, FOX, EBOX, SP1/KLF, ETS, HOMEOBOX |
| New 3 | FOX, EBOX, ETS |
| New 4 | SRY, FOX, SP1/KLF, ETS, HOMEOBOX |

**Figure 14.5** Finding potential cis-regulatory elements. (A) ChIP-seq signal tracks for H3K4me1 and H3K27ac in a 50 kb area on either side of the transcription start site of the *COL2A1* gene. Validated (green) and new regulatory areas (red) are highlighted. (B) Left side: motif scanning for TF-binding sites in regions enriched for H3K27ac in differentiated cells. An example motif from the Jaspar database is shown. The members of the TF families are those known to be involved in chondrogenesis that share similar binding motifs. Right side: motif hits in regulatory areas around the *COL2A1* gene. (See color plate section.) *Source*: [52] Herlofsen, S.R. *et al*., *BMC Genomics* 2013; **14**, 105.

these genes. One important exception is *COL10A1*. Here, most CpG sites are methylated, while two sites are partially methylated in the promoter region of undifferentiated MSCs. These sites were less methylated after differentiation. As no restrictive histone modifications are present in the *COL10A1* promoter in undifferentiated MSCs, the DNA methylation is likely to contribute to the lack of *COL10A1* transcription in the undifferentiated state, while the reduced DNA methylation in the two CpG sites may facilitate *COL10A1* transcription in the course of differentiation. Changes in histone marks can reproducibly be used to predict changes in gene transcription, with H3K36me3 the mark most closely associated with active transcription. Increases in promoter marks H3K4me3 and H3K9ac may identify genes that are prepatterned for transcription before active transcription occurs. Histone marks H3K4me1 and H3K27ac may be used to identify active enhancer regions.

## 14.9 Role of microRNAs during early *in vitro* chondrogenesis

### 14.9.1 An introduction to microRNAs

MicroRNAs (miRNAs) are small double-stranded RNA molecules that regulate gene expression by targeting complementary nucleic acid sequences (Figure 14.6). miRNAs are transcribed as large transcripts called primary miRNA (pri-miRNA). In the nucleus, pri-miRNAs are recognized and processed by the nuclease Drosha [55]. The resulting product is called precursor miRNA (pre-miRNA) and contains a two-nucleotide overhang at its 3′ end. The Exportin complex then transports the pre-miRNA into the cytoplasm, where another nuclease, Dicer, cleaves the pre-miRNA into mature double-stranded miRNA molecules approximately 21–23 nucleotides in length [56]. One or both of the miRNA strands are incorporated into the RNA-induced silencing complex where they can bind to complementary mRNA

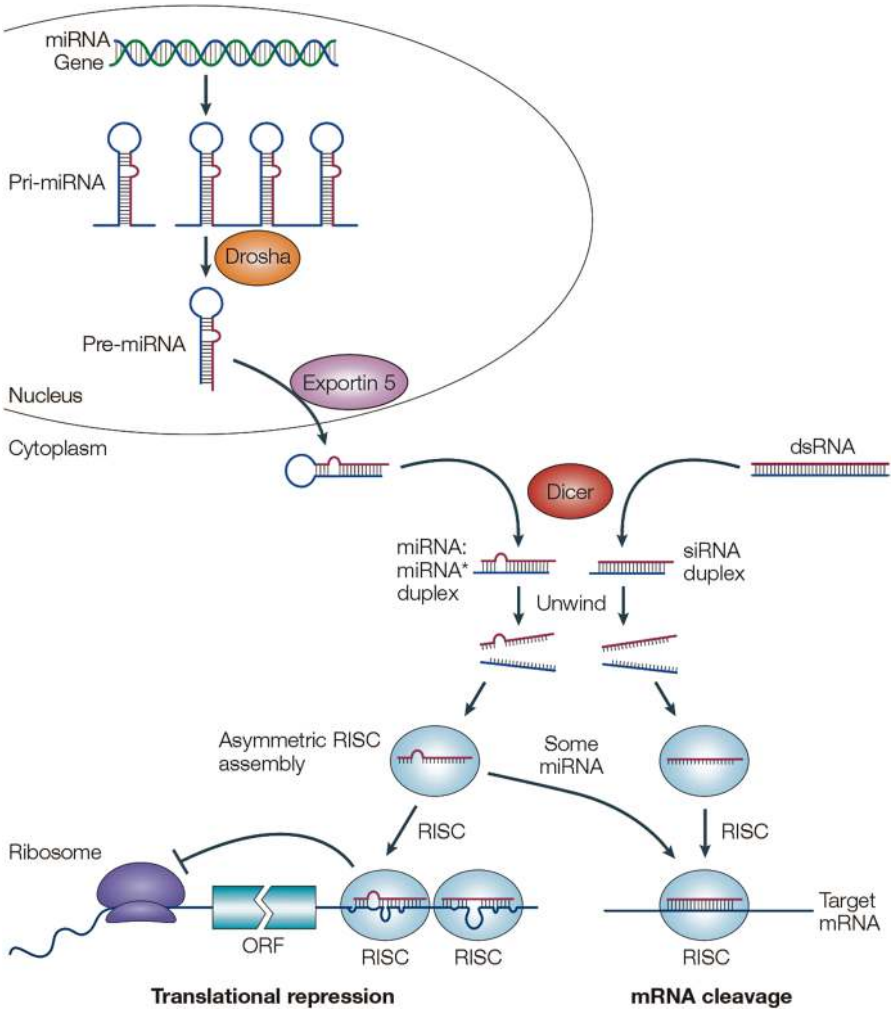



**Figure 14.6** MicroRNA biogenesis. The nascent pri-microRNA (pri-miRNA) transcripts are first processed into 70-nucleotide pre-miRNAs by Drosha inside the nucleus. Pre-miRNAs are transported to the cytoplasm by Exportin 5 and are processed into miRNA duplexes by Dicer. One strand (usually) of the miRNA duplex is assembled into the RNA-induced silencing complex (RISC), which subsequently acts on its target by translational repression or mRNA cleavage, depending, at least in part, on the level of complementarity between the small RNA and its target. ORF: open reading frame. *Source*: [54] He L and Hannon GJ 2004, p. 522. Reproduced with permission of Nature Publishing Group.

molecules by base-pairing, resulting in either degradation of the mRNA or translational repression [57,58]. The two miRNA strands are sometimes given the suffix 5p and 3p, which indicates whether the strand is derived from the 5′ or the 3′ arm of the pri-miRNA. The nucleotides at positions 2 to 8 at the 5′ end of the mature miRNA strand are called the seed sequence and have been demonstrated to be crucial for recognizing mRNA target sequences. It is believed that most target sequences are found in the 3′ untranslated region (UTR) of the mRNA, but miRNAs can also target sequences in the 5′ UTR coding regions and in promotor regions [59–61].

Most studies have demonstrated that miRNAs predominantly downregulate gene expression, but miRNAs have also been shown to stimulate gene expression. This may occur through activation of translation [62,63] or by targeting complementary sequences in promotor regions to induce transcription [60,64]. Because the seed sequence is short, one miRNA has the potential to regulate many genes. Several studies have shown that a single miRNA can affect the expression of hundreds of genes, directly or indirectly [65–67]. More than 2500 miRNAs have been discovered in humans so far (www.miRbase.org), and it has been estimated that 60% of human genes are regulated by miRNAs [68]. This indicates that most biological processes are regulated by miRNAs.

### 14.9.2 Role of miRNA-140 in developmental chondrogenesis

Deletion of the miRNA processing enzyme Dicer will greatly reduce or abolish production of most mature miRNAs. Cartilage-specific knockout of Dicer in mice leads to reduced chondrocyte proliferation and accelerated hypertrophic differentiation, resulting in severe skeletal defects and premature death [69]. These results show that miRNAs are important regulators of cartilage biology. Several miRNAs have been investigated during chondrogenesis. Perhaps the best understood and most studied miRNA in cartilage biology is miRNA-140 (miR-140). miR-140

is considered to be a cartilage-specific miRNA as its expression is restricted to cartilaginous tissues during embryonic development in mouse and zebrafish [70,71]. Whole mount *in situ* hybridization demonstrates overlapping expression of miR-140 with *SOX9* and *COL2a1* in mouse limb buds during development [72,73]. Miyaki *et al.* showed that miR-140 was expressed in proliferating chondrocytes in the growth plate but not in hypertrophic chondrocytes. In addition, miR-140-deficient mice had fewer proliferating chondrocytes, indicating that the skeletal phenotype was due to reduced chondrocyte proliferation in the growth plate [74]. In fact, miR-140 knockout mice show similarities with cartilage-specific Dicer knockout mice, indicating that miR-140 is one of the most important miRNAs for normal cartilage and skeletal development.

### 14.9.3 miR-140 targets identified *in vivo* and in vitro

Reduced chondrocyte proliferation may be a result of miR-140 targeting the TF SP1, as SP1 inhibits cell cycle progression and has been shown to be a target of miR-140 in micromass cultures [72,75]. In another *in vivo* study, Nakamura *et al.* showed impaired endochondral bone formation and enhanced hypertrophic differentiation in miR-140 knockout mice. It was demonstrated that miR-140 targeted DNPEP (aspartyl aminopeptidase), which modulates BMP signaling [76]. A study in zebrafish embryos showed that inhibiting miR-140 led to craniofacial defects such as clefting of the cartilage structures in the mouth of the zebrafish larvae. Platelet-derived growth factor-α (*PDGF-α*) was validated as a target of miR-140, and reduced PDGF-α was identified as the cause of the defect [77]. In a mouse model of antigen-induced arthritis, Miyaki *et al.* demonstrated that overexpression of miR-140 in cartilage ameliorated the arthritis phenotype by reducing the loss of PGs and COL2, while miR-140 knockout mice were predisposed to OA. The aggrecan-degrading enzyme ADAMTS5 (a disintegrin and metalloproteinase with thrombospondin motif 5) was identified as a direct target of miR-140, partially explaining the protective role of miR-140 against OA progression [74].

SMAD3 is a key TF in transforming growth factor (TGF)-β signaling and has been shown to be a target of miR-140 in C3H10T1/2 cells (a multipotential mesenchymal cell line derived from C3H mice) [78]. Other validated targets of miR-140 are *BMP2*, *CXCL12*, and *HDAC4* [71,79,80]. BMP2 is a member of the TGF superfamily and is important for chondrogenesis and bone formation [81]. Overexpression of *BMP2* in developing chick limbs led to increased number of chondrocytes and increased deposition of ECM [82,83]. miR-140 may therefore regulate proliferation and ECM production via BMP2. CXCL12 is a ligand for the CXCR4 receptor and regulates several physiological functions, such as angiogenesis and migration of several cell types, including MSCs [84]. During murine embryogenesis CXCL12 is synthesized by prehypertrophic and hypertrophic chondrocytes and stimulates bone growth by modulating the actin cytoskeleton [85]. CXCL12 is also elevated in the synovial fluid of OA patients and enhances proliferation and COL1 synthesis in osteoblasts from OA patients [86,87]. During *in vitro* chondrogenesis of human MSCs the expression of *CXCL12* shows an inverted relationship with miR-140 [67,88]. Thus, miR-140 induced downregulation of CXCL12 may in several ways be beneficial for chondrogenesis. Finally, the miR-140 target *HDAC4* encodes a histone deacetylase that modulates the TF RUNX2, an important regulator of hypertrophic differentiation [89].

### 14.9.4 Defining the role of miR-140 during chondrogenic differentiation of mesenchymal stromal cells and dedifferentiation of articular chondrocytes

In 2011 our group published the first study investigating global miRNA changes during dedifferentiation of human ACs. Several miRNAs decreased during dedifferentiation, including miR-140 [90]. We later compared these results with those obtained during *in vitro* chondrogenic differentiation of MSCs [67]. miR-140 was the most highly expressed miRNA in chondrogenically differentiated MSCs and in uncultured ACs. Comparing expression changes of all miRNAs, miR-140 was both the most upregulated miRNA during chondrogenesis of MSCs and the most downregulated miRNA during dedifferentiation of ACs (Figure 14.7 and Table 14.1). Thus, both *SOX9* and miR-140 increase during chondrogenic differentiation of MSCs. This may reflect the regulatory role played by SOX9 on miR-140 transcription. miR-140 is an intronic miRNA located in the *WWP2* (WW domain containing E3 ubiquitin protein ligase 2) gene and it is activated by SOX9 [72,91]. SOX5 and SOX6 also enhance expression of miR-140 by facilitating dimerization and DNA binding of SOX9 to the miR-140 promoter region [73].

In order to be able to describe the miR-140 interactome during *in vitro* chondrogenesis we inhibited miR-140 by introducing an anti-miR-140 into MSCs by lentiviral transduction [67]. (An interactome is the whole set of molecular interactions in a particular cell.) Gene ontology analysis of differentially expressed genes between miR-140 inhibited cells and control cells subsequently exposed to chondrogenic differentiation showed that inhibition of miR-140 during chondrogenesis led to decreased expression of genes that were mainly related to ECM, while the upregulated genes were mainly involved in the cell cycle and the cytoskeleton. These results were mirrored in dedifferentiating chondrocytes overexpressing miR-140 [67]. *ACAN* and *SOX9* mRNAs were not differently expressed in miR-140 inhibited cells, but the proteins were strongly reduced, suggesting a translational effect of miR-140 on these molecules. miR-140 inhibition also resulted in reduced synthesis of GAGs. In the same study we identified *RALA* (v-ral simian leukemia viral oncogene homolog A), a small GTPase, as a direct target of miR-140. Small interfering (siRNA)-mediated knockdown of *RALA* reduced SOX9 protein, suggesting that miR-140 works by inhibiting *RALA*, which is an inhibitor of *SOX9* translation. The net

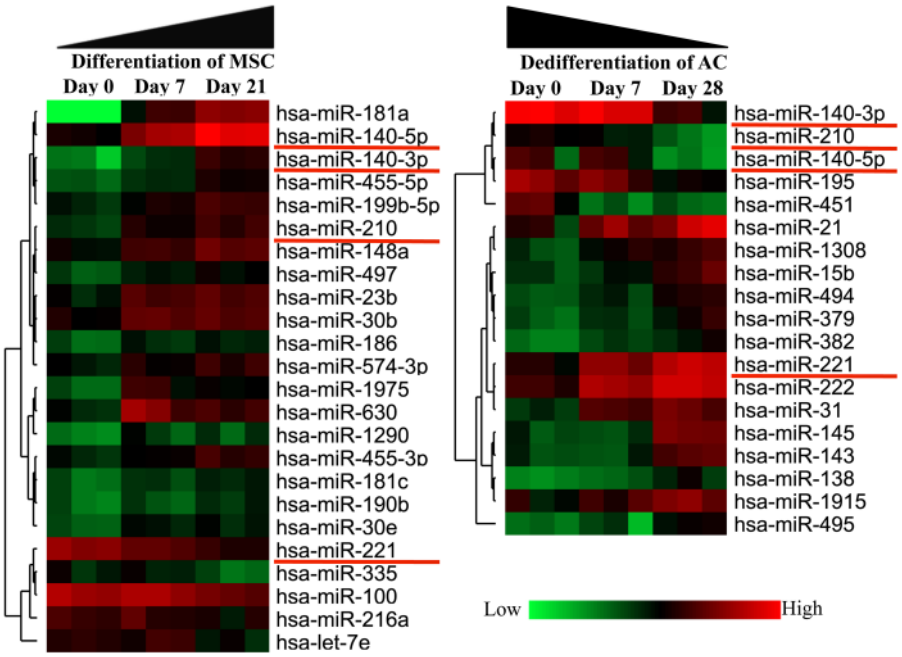



**Figure 14.7** miRNA profile relationship between differentiating MSCs and dedifferentiating ACs. Heat maps of differentially expressed miRNAs in differentiating MSCs and dedifferentiating ACs ($n = 3$ for each time point, false discovery rate <0.05). Reciprocally regulated miRNAs are underlined. (See color plate section.) *Source*: [67] Karlsen TA *et al.*, *Stem Cell Dev.* 2014; **23**: p. 293.

effect is that upregulation of miR-140 leads to upregulation of SOX9, which stimulates chondrogenesis and maintains cartilage homeostasis. We also found potential binding sites for miR-140 in the 5′ UTR of *SOX9* and *ACAN*, suggesting a potential binding of miR-140 to these mRNAs. As explained earlier, 5′ binding of miRNAs to their target mRNAs has been shown to enhance translation, and this could be another as yet unknown function of miR-140.

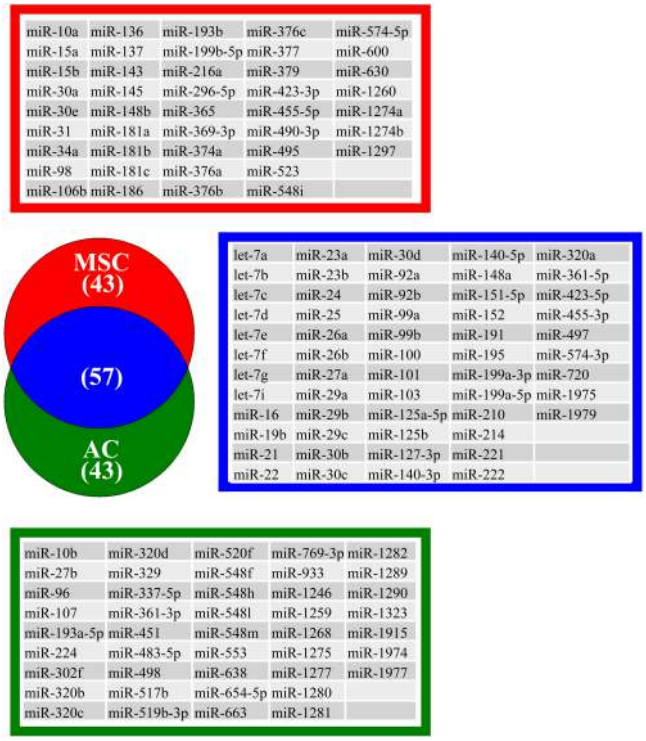
### 14.9.5 Impact of microRNAs other than miR-140 on chondrogenic differentiation of mesenchymal stromal cells

In addition to miR-140, other miRNAs were differently expressed at days 1, 3, 7, and 21 during chondrogenesis of MSCs [67]. Several of these miRNAs have already been associated with cartilage biology, including the upregulated miRNAs miR-16, 21, 23b, 26a, 30a, 30b, 148a, 181a, 199b, 210, 222, 455-3p, 630, and 1290 and the downregulated miR-100, 221, and 335 (Figure 14.8 and Table 14.1). Elevated levels of miR-16 have been associated with OA [92]. The upregulation of miR-16 may therefore be unwanted, as this could potentially promote OA progression. miR-21 is associated with fibrosis in several tissues, and inhibition of miR-21 leads to reduced COL1 production [93]. Upregulation of miR-21 is common for both differentiating MSCs (day 7) and dedifferentiating ACs (Figure 14.8 and Table 14.1). Both cell types produce COL1, consistent with a role for miR-21 in fibrocartilage formation during *in vitro* and *in vivo* chondrogenesis [88,94]. In addition, miR-21 is upregulated in OA cartilage, where it targets *GDF5*, the gene encoding the factor involved in the differentiation of the hyaline cartilage precursor interzone cells [95]. Thus, the upregulation of miR-21 may be involved in COL1 synthesis during chondrogenesis of MSCs and, therefore, may be one of the factors leading to fibrocartilage formation after ACI. Downregulation of *GDF5* may reduce the likelihood of producing hyaline cartilage as repair tissue in OA. Overexpression of miR-23b facilitates chondrogenic differentiation of synovial-fluid-derived MSCs by inhibiting protein kinase A. miR-23b also decreased the expression of *MMP2* and *MMP9* [96]. miR-26a is differently expressed at different developmental stages in rat cartilage [97] and is one of the miRNAs shown to be upregulated in OA cartilage [92]. miR-199b and miR-30a are upregulated during chondrogenic differentiation of murine MSCs [98,99]. miR-30a has been found to be essential for normal tracheal hyaline chondrocyte differentiation. Inhibition of miR-30a in tracheal chondrocytes reduced both ACAN and COL2 deposition [100]. miR-30b is upregulated in OA cartilage [92]. Upregulation of miR-148a may be beneficial since overexpression of miR-148a promotes cartilage formation and inhibits hypertrophic differentiation and degradation in OA chondrocytes [101]. miR-181a has been shown to inhibit *ACAN* and *CCN1*. The net effect is inhibition of chondrogenesis and promotion of osteoblast differentiation [102,103]. miR-210 is a so-called "hypoxic miRNA" because it is induced under hypoxic conditions [104]. miR-210 is also upregulated during chondrogenesis of adipose tissue-derived MSCs and has recently been shown to prevent cartilage degradation and to stimulate *COL2A1* in rat meniscus [105,106]. miR-210 is also downregulated in OA cartilage and in dedifferentiated chondrocytes compared to uncultured ACs (Figure 14.8) [92], indicating a positive effect of miR-210 on chondrogenesis. miR-222 expression is higher in weight-bearing cartilage compared with non-weight-bearing cartilage, suggesting miR-222 to have a role in

**Table 14.1** Significantly up- and downregulated miRNAs in MSCs undergoing chondrogenic differentiation [67].

| Day 1 | Mean expression | Fold change from control | Day 3 | Mean expression | Fold change from control | Day 7 | Mean expression | Fold change from control | Day 21 | Mean expression | Fold change from control |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Upregulated* | | | | | | | | | | | |
| hsa-miR-1974 | 249 | 8.1 | hsa-miR-630 | 1 577 | 15.3 | hsa-miR-630 | 2 171 | 21.0 | hsa-miR-140-5p | 14 821 | 62.0 |
| hsa-miR-1975 | 158 | 5.5 | hsa-miR-1974 | 244 | 11.1 | hsa-miR-1975 | 416 | 14.3 | hsa-miR-140-3p | 418 | 34.8 |
| hsa-miR-1290 | 74 | 5.2 | hsa-miR-1975 | 320 | 8.0 | hsa-miR-140-5p | 3 285 | 13.7 | hsa-miR-455-5p | 263 | 8.9 |
| | | | | | | hsa-miR-21 | 29 146 | 9.4 | hsa-miR-210 | 532 | 8.7 |
| | | | | | | hsa-miR-1290 | 83 | 5.8 | hsa-miR-199b-5p | 587 | 6.3 |
| | | | | | | hsa-miR-140-3p | 65 | 5.4 | hsa-miR-148a | 1 094 | 6.2 |
| | | | | | | hsa-miR-1974 | 150 | 4.9 | hsa-miR-1975 | 180 | 6.2 |
| | | | | | | hsa-miR-210 | 277 | 4.5 | hsa-miR-23b | 821 | 6.1 |
| | | | | | | hsa-miR-30a | 302 | 4.5 | hsa-miR-630 | 589 | 5.7 |
| | | | | | | hsa-miR-23a | 2 778 | 4.3 | hsa-miR-497 | 189 | 4.8 |
| | | | | | | hsa-miR-30b | 1 024 | 4.3 | hsa-miR-455-3p | 496 | 4.6 |
| | | | | | | hsa-miR-222 | 1 149 | 4.2 | hsa-miR-1290 | 59 | 4.1 |
| | | | | | | hsa-miR-26a | 4 836 | 4.0 | hsa-miR-30b | 927 | 3.9 |
| | | | | | | hsa-miR-30e | 123 | 3.8 | hsa-miR-30e | 120 | 3.8 |
| | | | | | | hsa-miR-95 | 53 | 3.6 | hsa-miR-574-3p | 490 | 3.8 |
| | | | | | | hsa-miR-193a-3p | 157 | 1.6 | hsa-miR-181c | 98 | 3.6 |
| | | | | | | hsa-miR-181a | 413 | N/A | hsa-miR-186 | 103 | 3.4 |
| | | | | | | hsa-miR-16 | 1 034 | N/A | hsa-miR-190b | 80 | 3.3 |
| | | | | | | hsa-miR-1246 | 270 | N/A | hsa-miR-181a | 2 310 | N/A |
| *Downregulated* | | | | | | | | | | | |
| None | | | hsa-miR-193a-3p | 19 | 5.3 | hsa-miR-221 | 972 | 2.6 | hsa-miR-221 | 374 | 6.6 |
| | | | hsa-miR-1274a | 666 | 1.8 | | | | hsa-miR-335 | 29 | 4.6 |
| | | | | | | | | | hsa-miR-100 | 1 222 | 3.1 |
| | | | | | | | | | hsa-let-7e | 132 | 2.8 |
| | | | | | | | | | hsa-miR-216a | 254 | 2.2 |

Statistics for assuming significance: rank product was used to compute the false discovery rate; cut-off set at <0.10 for days 1, 3, and 7 and <0.05 for day 21.




| | | | | |
|---|---|---|---|---|
| miR-10a | miR-136 | miR-193b | miR-376c | miR-574-5p |
| miR-15a | miR-137 | miR-199b-5p | miR-377 | miR-600 |
| miR-15b | miR-143 | miR-216a | miR-379 | miR-630 |
| miR-30a | miR-145 | miR-296-5p | miR-423-3p | miR-1260 |
| miR-30e | miR-148b | miR-365 | miR-455-5p | miR-1274a |
| miR-31 | miR-181a | miR-369-3p | miR-490-3p | miR-1274b |
| miR-34a | miR-181b | miR-374a | miR-495 | miR-1297 |
| miR-98 | miR-181c | miR-376a | miR-523 | |
| miR-106b | miR-186 | miR-376b | miR-548i | |

| | | | | |
|---|---|---|---|---|
| let-7a | miR-23a | miR-30d | miR-140-5p | miR-320a |
| let-7b | miR-23b | miR-92a | miR-148a | miR-361-5p |
| let-7c | miR-24 | miR-92b | miR-151-5p | miR-423-5p |
| let-7d | miR-25 | miR-99a | miR-152 | miR-455-3p |
| let-7e | miR-26a | miR-99b | miR-191 | miR-497 |
| let-7f | miR-26b | miR-100 | miR-195 | miR-574-3p |
| let-7g | miR-27a | miR-101 | miR-199a-3p | miR-720 |
| let-7i | miR-29a | miR-103 | miR-199a-5p | miR-1975 |
| miR-16 | miR-29b | miR-125a-5p | miR-210 | miR-1979 |
| miR-19b | miR-29c | miR-125b | miR-214 | |
| miR-21 | miR-30b | miR-127-3p | miR-221 | |
| miR-22 | miR-30c | miR-140-3p | miR-222 | |

| | | | | |
|---|---|---|---|---|
| miR-10b | miR-320d | miR-520f | miR-769-3p | miR-1282 |
| miR-27b | miR-329 | miR-548f | miR-933 | miR-1289 |
| miR-96 | miR-337-5p | miR-548h | miR-1246 | miR-1290 |
| miR-107 | miR-361-3p | miR-548l | miR-1259 | miR-1323 |
| miR-193a-5p | miR-451 | miR-548m | miR-1268 | miR-1915 |
| miR-224 | miR-483-5p | miR-553 | miR-1275 | miR-1974 |
| miR-302f | miR-498 | miR-638 | miR-1277 | miR-1977 |
| miR-320b | miR-517b | miR-654-5p | miR-1280 | |
| miR-320c | miR-519b-3p | miR-663 | miR-1281 | |

**Figure 14.8** Venn diagram with corresponding tables of the 100 highest expressed miRNAs in differentiated MSCs and uncultured ACs. (See color plate section.) *Source*: [67] Karlsen TA *et al.*, *Stem Cell Dev.* 2014; **23**: p. 293.

mechanotransduction [107]. miR-455-3p is upregulated during chondrogenesis of AT-MSCs and has been shown to be upregulated in human OA cartilage [106,108]. On day 3 and day 7 of *in vitro* chondrogenesis miR-630 was the most upregulated miRNA (Table 14.1). This is consistent with recent findings linking miR-630 to chondrogenesis [109]. In the same study, miR-1290 was also upregulated during chondrogenesis while miR-100 was downregulated. No known function of miR-1290 and miR-100 in cartilage biology has been identified so far. Downregulation of miR-221 during chondrogenesis is consistent with a publication demonstrating upregulation of *COL2A1* and *SOX9* after inhibition of miR-221 during MSC chondrogenesis [110]. miR-221 has also been shown to regulate chondrogenesis in chick limb MSCs [111]. We also observed downregulation of miR-335 during chondrogenesis. This is in line with earlier findings that downregulation of miR-335 is critical in MSC differentiation [112].

Some of the miRNAs discussed here are associated with OA or have other unwanted functions. This should be kept in mind when using MSCs for chondrogenesis and tissue engineering purposes. MSCs differentiated to chondrocytes should be as similar to native cartilage as possible. This includes miRNA expression profiles. In our study we compared the 100 most highly expressed miRNAs in uncultured ACs and differentiated MSCs and found a 57% overlap of shared miRNAs (Figure 14.8). We found that the nonoverlapping miRNAs highly expressed in differentiated MSCs were either not expressed or barely detectable in uncultured ACs, and the nonoverlapping miRNAs highly expressed in uncultured ACs were either not expressed or barely detectable in differentiated MSCs [67]. This shows that differentiated MSCs may express several unwanted miRNAs and lack expression of many wanted miRNAs in order to be regarded as true chondrocytes. For example, miR-181a is highly expressed in chondrogenically differentiated MSCs but has a very low expression in uncultured ACs. This miRNA was described earlier to be inhibitory of chondrogenesis by downregulating *ACAN* and *CCN1*.

Several miRNAs previously associated with cartilage and chondrogenesis were not differently expressed in our experiments using MSCs. However, some of the nondifferently expressed miRNAs had a very high or very low expression in MSCs, and several changed during dedifferentiation of ACs. miR-145 is highly upregulated during dedifferentiation of ACs and highly expressed by MSCs (Figures 14.8 and 14.9). *SOX9* has been verified as a target of miR-145 in two studies demonstrating an inhibiting effect of miR-145 in chondrogenesis and cartilage formation [107,113]. miR-145 is also increased in IL-1β-treated chondrocytes and in OA chondrocytes, where it modulates *SMAD3* [114]. Thus, suppressing miR-145 expression may be beneficial for tissue engineering of cartilage. Overexpression of miR-675 positively regulates COL2 synthesis, possibly via a SOX9-dependent mechanism [115]. miR-199a inhibits early BMP2-induced chondrogenesis in C3H10T1/2 cells by targeting *SMAD1* [116]. In another study, miR-1 was found to be repressed during hypertrophic differentiation and to inhibit the expression of *ACAN* [117]. miR-365 has been shown to be highly expressed in hypertrophic chondrocytes and to stimulate COL10 synthesis [118]. miR-365 is also highly expressed in MSCs but not expressed in uncultured ACs (Figure 14.9) [67]. miR-138 and miR-495 both increase during AC dedifferentiation (Figure 14.8 and Table 14.1). In one study, miR-138 was found to be overexpressed in differentiated and hypertrophic chondrocytes compared with precursor cells [119]. In other studies, miR-138 inhibited osteogenic and adipogenic differentiation [120,121], while miR-495 targeted *SOX9* and inhibited chondrogenesis in human MSCs [122].

### 14.9.6 MicroRNAs in chondrogenesis: conclusions

A wide range of miRNAs have been shown to be involved in chondrogenesis in different model systems. miR-140 seems to be the single miRNA most prominently promoting chondrogenesis, both *in vitro* and *in vivo*. In addition, miR-140 may have a protective role in arthritis. A number of mRNAs have been shown to be targets for miR-140, but none of them seem, on their own, to be able to explain the important role played by miR-140. We have recently demonstrated that miR-140 may induce translational upregulation of SOX9. The mechanism is not fully identified, but the prominent role played by SOX9 both for

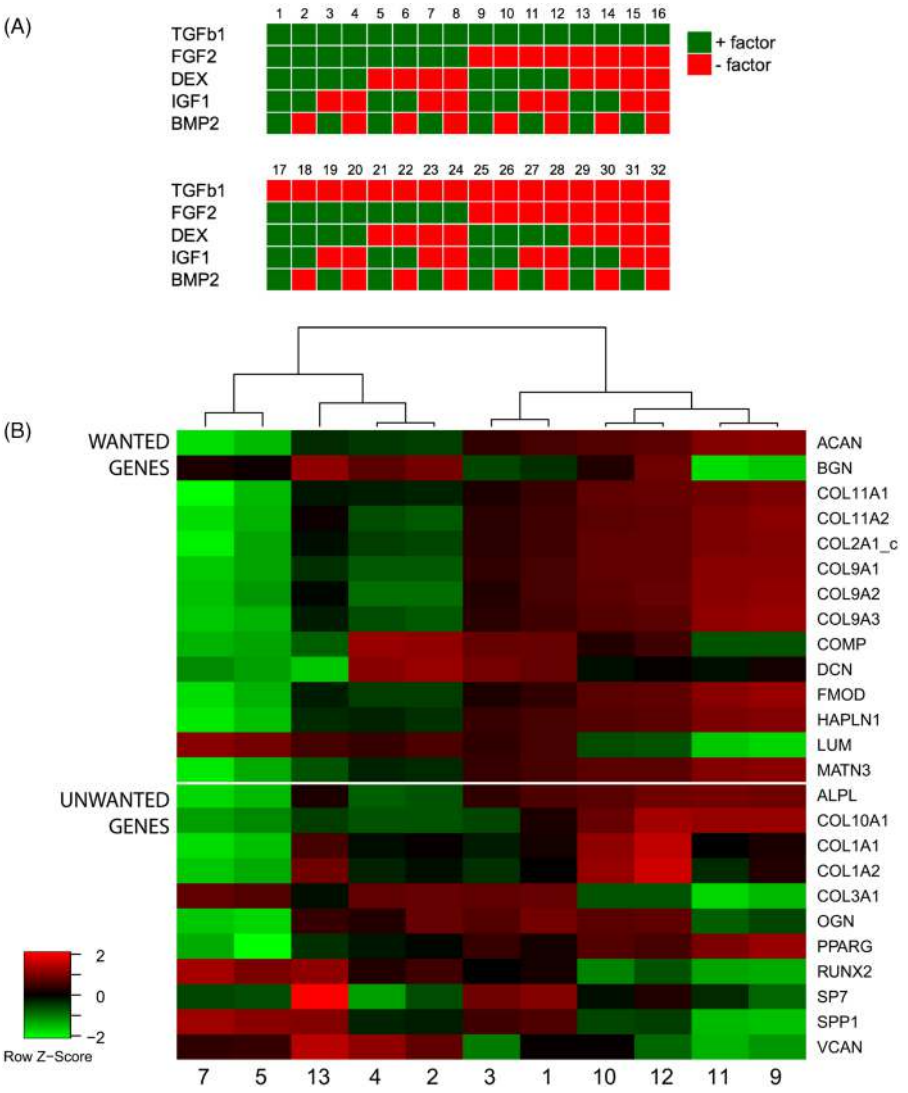



**Figure 14.9** (A) Experimental setup with numbering of the different conditions in a factorial analysis of five factors relevant to chondrogenesis. (B) Gene set enrichment analysis. Heat map of the expression of wanted and unwanted genes in the top ranking conditions significantly enriched for wanted genes (false discovery rate <0.05) but not enriched for unwanted genes. Color coded by the Studentized score. (See color plate section.) *Source*: [37] Jakobsen, *PLoS ONE* 2014, **9**: e96615.

chondrogenesis and OA suggests that it must be mechanistically important. A number of other miRNAs are differentially regulated during *in vitro* chondrogenic differentiation of MSCs. Some of these have been shown to be highly expressed in uncultured ACs and to be reduced during dedifferentiation. These are probably miRNAs with beneficial effects. Other miRNAs are not found in ACs, suggesting that they are not required in functional ACs. As miRNAs have regulatory effects on a wide range of genes, the role played by these potentially unwanted miRNAs requires further investigation.

## 14.10 Early changes in gene expression during *in vitro* chondrogenesis

The expression of key chondrogenesis genes has been reported in hundreds of papers. For biological relevance one needs to look at all or most of the genes that are essential for the formation of hyaline cartilage: genes involved in COL2 fibrillogenesis, in the synthesis if the major PGs and GAGs, some of the other major components of the cartilage ECM, and some of the unwanted genes expressed in fibrocartilage, in hypertrophic cartilage, and in bone formation. We studied changes in gene expression when MSCs were placed in alginate discs and exposed to a differentiation cocktail of TGF-β1 10 ng/mL, BMP2 500 ng/mL, and 0.1 μM dexamethasone for 3 weeks. The data were collected by microarray analysis at 0, 7, 14 and 21 days for cells from three donors, in some cases confirmed by real-time quantitative polymerase chain reaction and immunohistochemical stainings [88]. We also have unpublished data in gene expression analysis at earlier time points on days 0, 1, 3, and 7 using the NanoString nCounter system where 350 custom selected genes were analyzed at each time point in cells from three donors using a similar chondrogenic differentiation system. For both these datasets we have control results from the same time points of cells in alginate discs without differentiation factors. We will shortly discuss below

changes in gene expression that are influenced by the composition of the differentiation cocktail.

### 14.10.1 Genes involved in collagen fibrillogenesis

The signature gene of hyaline cartilage, *COL2A1*, is not expressed in MSCs. This makes results describing fold change increases in gene expression relative to day 0 difficult to interpret. *COL2A1* mRNA begins to increase between day 3 and day 7, is almost at peak expression by day 14, and by day 21 is one of the most highly expressed genes. The IIB splice variant is expressed at a very much higher level than the embryological IIA variant, but both are expressed. *COL9A2* and *COL9A3* are expressed from day 3, with maximal expression from day 14. However, *COL9A1*, which is essential for COL9 heterotrimer formation and thus for functionality, only increases between days 7 and 14 and is expressed at lower levels than the *A2* and *A3* isoforms. *COL11A1* is expressed at low levels in MSCs, increases from day 3, and is expressed at moderate levels on day 21. In contrast, *COL11A2* is not expressed in MSCs, is expressed on day 7, but is expressed at lower levels than its isoform counterpart on day 21. Other molecules of importance for the formation of COL2 fibrils are the small leucine-rich PGs decorin (*DCN*), biglycan (*BGN*), fibromodulin (*FMOD*), and lumican (*LUM*). *DCN* is expressed in MSCs, increases by day 1, and is highly expressed from day 3. *BGN* follows a similar pattern but at lower levels of expression. *FMOD* is expressed at very low levels in MSCs, but increases throughout differentiation to reach high levels on day 21. *LUM* is expressed at moderately high levels in MSCs and does not change much during differentiation. Finally, for fibrillation to occur, procollagen propeptides at either end need to be cleaved off. C-proteinase activity is possessed by BMP1, while N-proteinase activity is provided by ADAMTS2, -3, and -14 [123]. *ADAMTS2* and *BMP1* are expressed at low to moderate levels in MSCs and do not change much during differentiation [124]. Thus, all the genes associated with COL2 fibrillogenesis are expressed in this model, albeit at different levels and with different kinetics. The slow onset of expression of COL9A1 may be the limiting factor for the onset of fibrillogenesis. One previous study of microarray analysis of gene expression in pellet differentiation cultures has been described. Where results from expression of the same genes were presented, they largely corroborated our findings [25].

### 14.10.2 Genes involved in synthesis of proteoglycans and glygosaminoglycans

The other major group of cartilage ECM molecules are the PGs and GAGs. Of these, aggrecan (*ACAN*) is expressed at low levels in MSCs and increases gradually from day 3 to reach moderately high expression levels on day 21. Hyaluronic acid is a high molecular weight polysaccharide synthesized by members of the hyaluronic acid synthase (HAS) family. *HAS1*, *HAS2*, and *HAS3* are all expressed in MSCs, *HAS3* at very low levels, and none of them change during *in vitro* chondrogenesis. Link protein links aggrecan to hyaluronic acid. Genes encoding link protein are hyaluronan and PG link protein (*HAPLN*) family members 1–4. Of these, *HAPLN1* and *HAPLN3* are expressed in MSCs. *HAPLN1* decreases after establishment in 3D culture, then increases to moderately high levels on day 21. *HAPLN3* remains at relatively low expression levels. An enzyme family of importance for the synthesis of the small GAGs that branch off from aggrecan is the xylosyltransferases. *XYLT1* and *XYLT2* are both expressed in MSCs at low levels. While *XYLT2* retains its expression level throughout chondrogenic differentiation, *XYLT1* increases a little. Finally, the surface lubricant lubricin, a PG encoded by the PG 4 gene (*PRG4)*, is marginally expressed in MSCs, increases somewhat by day 3, and then remains expressed at low levels. Together, these results show that gene expression changes during *in vitro* chondrogenesis. For optimal cell-based treatment it will be important to ensure that synthesis of all the major PGs and GAGs is present in the cells used.

### 14.10.3 Transcription factor genes

The TFs of greatest importance for transcription of many of the ECM molecules belong to the SOX group. *SOX9* is expressed throughout developmental chondrogenesis in mice and is found at high levels also in human prechondrocytes [7,8,10]. In our *in vitro* chondrogenesis assay *SOX9* mRNA is found in MSCs, increases about 10-fold by day 7 and then remains at moderately high expression levels throughout the assay. For complete generation of hyaline cartilage, SOX9 must interact with SOX5 and SOX6 [125]. In our differentiation assay *SOX5* and *SOX6* transcripts are either extremely low or absent in MSCs, are expressed at low levels on day 3, and remain at low levels throughout the assay. However, another member of the SOX family, SOX8 was found to rise from no expression in MSCs to low expression on day 7 and to moderate expression from day 14 onwards.

This TF is interesting, because it is one of the most highly upregulated TFs during murine developmental chondrogenesis and it is also found to be upregulated during human embryological chondrogenesis [8,10]. We studied the role of SOX8 in *in vitro* chondrogenesis [126]. Using siRNA inhibition assays we found that the expression of *SOX8* mRNA was regulated by SOX9 but not vice versa. Transfection with either siRNA greatly reduced the expression of a long list of mRNAs essential for chondrogenesis. such as *COL2A1*, *COL9A2* and *A3*, *COL11A1* and *A2*, *HAPLN1*, *ACAN*, and many others, suggesting that SOX8 and SOX9 cooperate in ensuring transcription of these molecules and that neither of them is redundant. Interestingly, for molecules involved in chondrocyte hypertrophy the situation was entirely different. *SOX8* inhibition did not seem to affect these at all. Inhibition of *SOX9* led to increased expression levels of *MMP13* and *ALPL* (alkaline phosphatase), with no change seen in *COL10A1* mRNA levels. These observations were confirmed by experiments with synthetic mRNAs [126]. This shows that SOX9 inhibits hypertrophy by inhibiting MMP13 and ALPL, while SOX8 does not seem to impact on the process of hypertrophy.

### 14.10.4 Genes encoding other important cartilage molecules

Other molecules are also important for the *in vitro* formation of hyaline cartilage. *COMP* was expressed at low levels in MSCs, increased considerably by day 7, and then remained expressed at slightly lower levels for the duration of the assay. COL6 is found in the immediate vicinity of the ACs. *COL6A1*, *-A2*, and *-A3* were all found to be expressed at moderately high levels in MSCs, and expression remained at approximately the same level throughout the assay. Fibronectin (*FN*) is a molecule thought to be of importance for collagen assembly [127]. *FN* is expressed at high levels in MSCs and with transcription it is one of the most abundant mRNAs in the cells by day 7. In order to exert its effect on collagen assembly, FN must bind to its cell receptor integrin α5β1. The mRNAs encoding these molecules – integrin α5 *(ITGA5*, also known as the fibronectin receptor, CD49e, and very late activation protein 5), and integrin β1 (*ITGB1*, also known as CD29, glycophorin IIa, and very late activation protein, β subunit) – are both expressed in MSCs at relatively high levels. During *in vitro* chondrogenesis *ITGA5* increases slightly and *ITGB1* is slightly reduced, but both are persistently expressed. It is thought that the COL2 integrin receptor α2β1 also needs to be expressed [127].

*ITGA2* (integrin α2, also known as CD49B, α2 subunit of the very late activation antigen-2 receptor, and platelet membrane glycoprotein Ia) is marginally expressed in MSCs, and expression is increased a little during the differentiation assay. It is not known if this low expression of the COL2 integrin receptor may be a hindrance for COL2 fibril formation. Apart from this, the vast majority of the molecules required for hyaline cartilage formation are expressed at the mRNA level.

### 14.10.5 Genes encoding unwanted molecules

Unfortunately, several unwanted molecules are also expressed. First, however, it is worth appreciating that a number of mRNAs that encode chemokines, cytokines, and ECM degradation molecules important for the role played by MSCs in inflammation are all downregulated as early events in chondrogenesis [52]. *COL1A1* and *-A2*, which ensure the synthesis of COL1, the most abundant collagen in fibrocartilage, are both expressed at high levels in MSCs. The expression is reduced considerably just by placing the MSCs in the alginate scaffold, but addition of the differentiation cocktail induces expression that is maintained at approximately the same level as in MSCs. Versican (*VCAN*) is the most important PG in fibrocartilage. *VCAN* is expressed at moderate levels in MSCs and is reduced, but not eliminated, in differentiating cells. Thus, the molecular basis for the formation of fibrocartilage is present side by side with the genes necessary for the formation of hyaline cartilage, although *COL2A1* and *ACAN* are expressed at 5–10 times higher levels than their fibrocartilage counterparts *COL1A1* and *VCAN* on day 21. Genes involved in chondrocyte hypertrophy are also expressed. *COL10A1*, which appears to be under different transcriptional control than *COL2A1*, is marginally expressed in MSCs, but already on day 1 significant mRNA levels are found, and levels similar to those observed for *COL2A1* are found on day 21. The MMPs most involved in COL2 breakdown are MMP13, MMP1, and MMP8. Of these, *MMP1* and *MMP13* are expressed at extremely low levels throughout differentiation, while *MMP8* is not expressed. Cleavage of aggrecan is mediated by the aggrecanases ADAMTS4 and ADAMTS5. Of these, *ADAMTS5* is expressed at very low levels in MSCs and then falls to near undetectable levels during differentiation, while *ADAMTS4* is not expressed at any time. Thus, the major chondrocyte hypertrophy catabolic enzymes are absent or only barely expressed during *in vitro* chondrogenesis. Another molecule involved in chondrocyte hypertrophy is *RUNX2*. This mRNA is present at low levels in MSCs and remains at unchanged levels throughout differentiation. Alkaline phosphatase (*ALPL)*, the gene product of which is responsible for deposition of calcium during bone formation, is expressed in MSCs, and increases between days 7 and 14 to moderately high levels. However, although genes involved in endochondral ossification are expressed at variable levels in our model of *in vitro* chondrogenesis, we have never seen evidence of bone formation in this model system.

### 14.10.6 Effect on gene expression of changes in the differentiation cocktail

The results presented here from our laboratory were all created using a differentiation cocktail containing TGF-β1, BMP2, and dexamethasone [128]. However, as our chondrogenic differentiation assay in alginate discs is different from the micromass cultures previously used to determine the optimal differentiation cocktail, we wanted to perform an optimalization study using our own chondrogenic differentiation assay. For this, we established a checkerboard factorial analysis involving TGF-β1, BMP2, dexamethasone, FGF2, and insulin-like growth factor 1 (IGF1). As a read-out system we used the custom-made NanoString nCounter assay, which allowed us to quantify 350 genes of our own selection for every condition established. Primary MSCs from three donors were used and data were collected from day 1 and day 7 [37]. First, however, we analyzed experiments to determine if any of the TGF-β isoforms TGF-β1, TGF-β2, or TGF-β3 performed better than the others in the presence or absence of dexamethasone or in the presence or absence of BMP2. Our conclusion was that there was no significant difference between these different isoforms, so we chose TGF-β1 for the remaining experiments. We also tested for differences between the use of BMP2, BMP4, or BMP6 in the presence or absence of TGF-β1, and since we did not discover important differences between these, we used BMP2 for the remaining experiments.

This discussion will be based predominantly on data from day 7. We had observed a trend toward the same results on day 1, suggesting that many of the important molecular events during *in vitro* chondrogenesis occur early, but robust distinguishing features between the differentiation cocktails tested were most obvious on day 7. It was clear that the factor with the greatest

influence on our gene set was TGF-β1. No other factor, regardless of the presence or absence of other factors, had the same effect on the upregulation of wanted chondrogenic genes. Unfortunately, addition of TGF-β1 also led to the upregulation of unwanted genes, but to a lesser extent. We concluded that TGF-β1 was indispensable in an *in vitro* chondrogenesis assay. As IGF1 has been described to have an influential role in developmental chondrogenesis we expected this factor to also have a positive influence in our *in vitro* chondrogenesis assay, but this was not the case. Analyzing lengthy lists of wanted and unwanted genes, we found that IGF was entirely neutral relative to the expression of these genes. FGF2 is a known stimulator of MSC proliferation, and the addition of FGF2 to the medium during the *in vitro* expansion stage has been shown to enhance subsequent chondrogenic differentiation [129,130]. In the present study, however, we only examined the effect of adding FGF2 to the differentiation cocktail. We found that FGF2 induced downregulation of both wanted and unwanted genes, both at relatively low magnitudes, but with a trend toward greater downregulation of wanted genes. The data can be visualized in Figure 14.10: compare conditions 1–4 (with FGF2) with conditions 9–12 (without FGF2). Of note, genes such as *COL2A1*, *COL9A1*, *-A2*, and *-A3*, and *COL11A1* and *-A2* were all downregulated in the presence of FGF2. On the other hand *COL10A1*, *PPAR-γ*, and to some extent *COL1A1* were also downregulated in the presence of FGF2. We concluded that FGF2 should not be part of our differentiation cocktail.

Corticosteroids have not been described as playing a role in embryological chondrogenesis, but dexamethasone has been included in protocols for *in vitro* chondrogenesis since the early studies of this process were performed [131]. In our own *in vitro* chondrogenesis assay it was obvious that addition of dexamethasone to TGF-β1 in the differentiation cocktail led to upregulation of all the wanted genes except for two of the small leucine-rich PGs (*BGN* and *LUM* – compare conditions 1 with dexamathasone and 5 without – but also to upregulation of some of the genes within our selection of unwanted genes, such as *COL10A1*, *COL1A1* and *-A2*, *ALPL*, *PPARG*, and *OGN* (osteoglycin). A more detailed inspection of the 20 most highly upregulated and downregulated genes following addition of dexamethasone revealed that the vast majority of the most highly

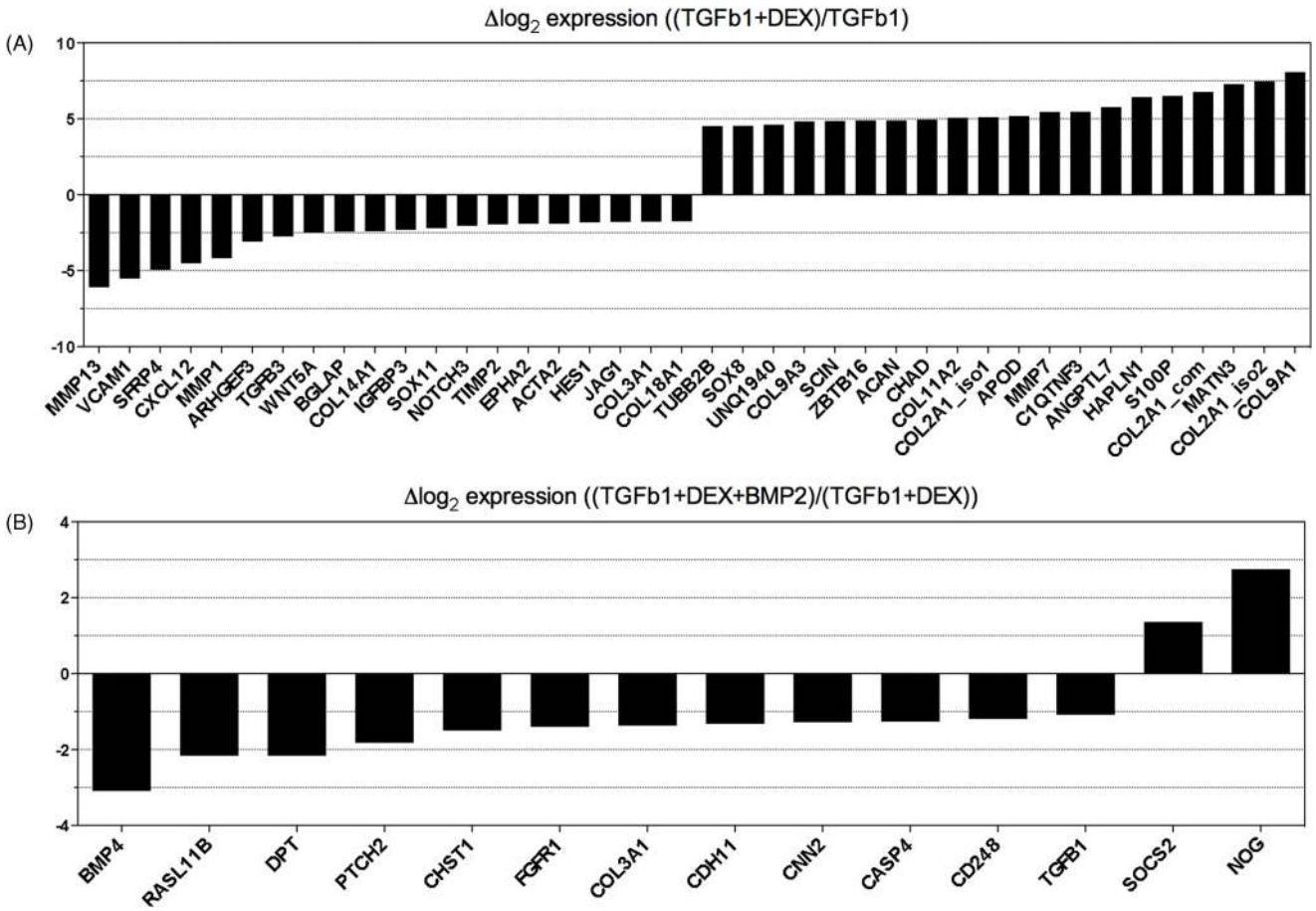



**Figure 14.10** Genes significantly regulated between key conditions (day 7). (A) Top 20 upregulated and top 20 downregulated genes when dexamethasone is added to TGF-β1. (B) Changes in gene expression when BMP2 is added to TGF-β and dexamethasone. Values represent log 2 to the fold change between the gene expression in the condition without and the condition with the specified factor added. *Source*: [37] Jakobsen, *PLoS ONE* 2014, **9**: e96615.

upregulated genes were likely to have a positive effect on *in vitro* chondrogenesis, while the majority of the downregulated genes were genes with gene products most likely to exert an unfavorable effect in our assay. Thus, dexamethasone was included in our differentiation assay.

With TGF-β1 and dexamathasone in place in the differentiation cocktail, we needed to determine whether to add BMP2 also. There were significantly upregulated or downregulated genes when BMP2 was added on a background of TGF-β1 and dexamethasone. The most highly upregulated gene, *NOG*, encodes the polypeptide NOGGIN, which binds and inactivates BMPs belonging to the TGF-β superfamily, particularly BMP4 [132]. This may be the reason why *BMP4* was the most downregulated gene, an effect that is presumably unwanted in this assay. Another downregulated gene was *NOTCH1*. The protein encoded by this gene has been shown to be required in early chondrogenesis, but must be turned off for complete chondrogenesis to occur [133]. In addition, several other downregulated genes, such as *DPT* (dermatopontin), *FGFR1* (FGF receptor 1), and TGF-β1, are likely to have prochondrogenic effects. We concluded that the addition of BMP2 to a chondrogenic cocktail already consisting of TGF-β1 and dexamethasone is not likely to improve cartilage formation.

## 14.11 Conclusions

The factors involved in developmental chondrogenesis in rodents are well described, and corresponding data from investigations in humans are accumulating [6,7,9,10]. These results have established a benchmark for scientists trying to make cartilage *in vitro*. The best approach so far is perhaps an assay that uses condensed masses from MSCs, potentially similar to the mesenchymal condensations observed during development. These condensations were press fitted into a porous decellularized bone matrix [45]. Although this model created cartilage of the right thickness and acceptable compressive capabilities, the system is probably too cumbersome for routine tissue engineering of cartilage for clinical use. For this, the use of MSCs in a biomaterial may still be the best choice. In this chapter we have discussed some of the molecular events occurring when MSCs are exposed to a chondrogenic differentiation cocktail in such a model system. We have shown that the vast majority of genes encoding proteins required for the synthesis of hyaline cartilage are expressed in this model. However, a number of unwanted genes are also expressed. Research is ongoing to find differentiation protocols that allow expression of all the wanted genes while avoiding the unwanted genes.

However, there are a number of other problems that need to be solved before the perfect implant can be developed. One issue is whether COL2 proteins are present as fibrils throughout the intercellular space. In hydrogel biomaterials this may not be the case [124]. Another issue is how important it is to achieve an overall architectural structure similar to that seen *in vivo*. This issue may perhaps not be solved before experimental therapies have been followed up for a number of years. Development of a perfect synthetic cartilage graft has the potential to help millions of people with OA and in so doing would also save a great deal of money currently spent on analgesics, joint surgery, or joint replacement. The book on this type of research is certainly not closed yet.

## References


1 Blevins, F.T., Steadman, J.R., Rodrigo, J.J., and Silliman, J. Treatment of articular cartilage defects in athletes: an analysis of functional outcome and lesion appearance. *Orthopedics*, 1998;**21**(7): 761–767, discussion 767–768.

2 Brittberg, M., Lindahl, A., Nilsson, A., *et al*. Treatment of deep cartilage defects in the knee with autologous chondrocyte transplantation. *N Engl J Med*, 1994;**331**(14):889–895.

3 Vasiliadis, H.S. and Wasiak, J. Autologous chondrocyte implantation for full thickness articular cartilage defects of the knee. *Cochrane Database Syst Rev*, 2010; (10):CD003323.

4 Holtzer, H., Abbot, J., Lash, J., and Hotzer, S. The loss of phenotypic traits by differentiated cells in vitro, I. Dedifferentiation of cartilage cells. *Proc Natl Acad Sci U S A*, 1960;**46**(12):1533–1542.

5 Matricali, G.A., Dereymaeker, G.P., and Luyten, F.P. Donor site morbidity after articular cartilage repair procedures: a review. *Acta Orthop Belg*, 2010;**76**(5):669–674.

6 Olsen, B.R., Reginato, A.M., and Wang, W. Bone development. *Annu Rev Cell Dev Biol*, 2000;**16**:191–220.

7 Goldring, M.B., Tsuchimochi, K., and Ijiri, K. The control of chondrogenesis. *J Cell Biochem*, 2006;**97**(1):33–44.

8 Cameron, T.L., Belluoccio, D., Farlie, P. G., *et al*. Global comparative transcriptome analysis of cartilage formation in vivo. *BMC Dev Biol*, 2009;**9**:20.

9 DeLise, A.M., Fischer, L., and Tuan, R.S. Cellular interactions and signaling in cartilage development. *Osteoarthritis Cartilage*, 2000; **8**(5):309–334.

10 Wu, L., Bluguermann, C., Kyupelyan, L., *et al*. Human developmental chondrogenesis as a basis for engineering chondrocytes from pluripotent stem cells. *Stem Cell Rep*, 2013;**1**(6):575–589.

11 Flannery, C.R., Hughes, C.E., Schumacher B.L., *et al*. Articular cartilage superficial zone protein (SZP) is homologous to megakaryocyte stimulating factor precursor and is a multifunctional proteoglycan with potential growth-promoting, cytoprotective, and lubricating properties in cartilage metabolism. *Biochem Biophys Res Commun*, 1999;**254**(3):535–541.

12 Hall, B.K. and Miyake, T. Divide, accumulate, differentiate: cell condensation in skeletal development revisited. *Int J Dev Biol*, 1995;**39**(6):881–893.

13 Hunziker, E.B. Mechanism of longitudinal bone growth and its regulation by growth plate chondrocytes. *Microsc Res Tech*, 1994;**28** (6):505–519.

14 Holder, N. An experimental investigation into the early development of the chick elbow joint. *J Embryol Exp Morphol*, 1977;**39**:115–127.

15 Koyama, E., Shibukawa, Y., Nagayama, M., *et al*. A distinct cohort of progenitor cells participates in synovial joint and articular cartilage formation during mouse limb skeletogenesis. *Dev Biol*, 2008; **316**(1):62–73.

16 Decker, R.S., Koyama, E., and Pacifici, M. Genesis and morphogenesis of limb synovial joints and articular cartilage. *Matrix Biol*, 2014;**39**:5–10.
17 Hyde, G., Dover, S., Aszodi, A., *et al*. Lineage tracing using matrilin-1 gene expression reveals that articular chondrocytes exist as the joint interzone forms. *Dev Biol*, 2007;**304**(2):825–833.
18 Strioga, M., Viswanathan, S., Darinskas, A., *et al*. Same or not the same? Comparison of adipose tissue-derived versus bone marrow-derived mesenchymal stem and stromal cells. *Stem Cells Dev*, 2012;**21**(14):2724–2752.
19 Jakobsen, R.B., Shahdadfar, A., Reinholt, F.P., and Brinchmann, J.E. Chondrogenesis in a hyaluronic acid scaffold: comparison between chondrocytes and MSC from bone marrow and adipose tissue. *Knee Surg Sports Traumatol Arthrosc*, 2010;**18**(10):1407–1416.
20 Liu, T.M., Martina, M., Hutmacher, D.W., *et al*. Identification of common pathways mediating differentiation of bone marrow- and adipose tissue-derived human mesenchymal stem cells into three mesenchymal lineages. *Stem Cells*, 2007;**25**(3):750–760.
21 Reinisch, A., Etchart, N., Thomas, D., *et al*. Epigenetic and *in vivo* comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood*, 2015;**125**(2):249–2604.
22 Lavrentieva, A., Hatlapatka, T., Neumann, A., *et al*. Potential for osteogenic and chondrogenic differentiation of MSC. *Adv Biochem Eng Biotechnol*, 2013;**129**:73–88.
23 Sakaguchi, Y., Sekiya, I., Yagishita, K., and Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum*, 2005;**52**(8):2521–2529.
24 Hubka, K.M., Dahlin, R.L., Meretoja, *et al*. Enhancing chondrogenic phenotype for cartilage tissue engineering: monoculture and coculture of articular chondrocytes and mesenchymal stem cells. *Tissue Eng Part B Rev*, 2014;**20**(6):641–654.
25 Sekiya, I., Vuoristo, J.T., Larson, B.L., and Prockop, D.J. *In vitro* cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc Natl Acad Sci U S A*, 2002;**99**(7):4397–4402.
26 Sotiropoulou, P.A., Perez, S.A., Salagianni, M., *et al*. Cell culture medium composition and translational adult bone marrow-derived stem cell research. *Stem Cells*, 2006;**24**(5):1409–1410.
27 Hagmann, S., Moradi, B., Frank, S., *et al*. Different culture media affect growth characteristics, surface marker distribution and chondrogenic differentiation of human bone marrow-derived mesenchymal stromal cells. *BMC Musculoskelet Disord*, 2013;**14**:223.
28 Selvaggi, T.A., Walker, R.E., and Fleisher, T.A. Development of antibodies to fetal calf serum with arthus-like reactions in human immunodeficiency virus-infected patients given syngeneic lymphocyte infusions. *Blood*, 1997;**89**(3):776–779.
29 Doerr, H.W., Cinatl, J., Stürmer, M., and Rabenau, H.F. Prions and orthopedic surgery. *Infection*, 2003;**31**(3):163–171.
30 Spees, J.L., Gregory, C.A., Singh, H., *et al*. Internalized antigens must be removed to prepare hypoimmunogenic mesenchymal stem cells for cell and gene therapy. *Mol Ther*, 2004;**9**(5):747–756.
31 Sensebe, L., Gadelorge, M., and Fleury-Cappellesso, S. Production of mesenchymal stromal/stem cells according to good manufacturing practices: a review. *Stem Cell Res Ther*, 2013;**4**(3):66.
32 Shahdadfar, A., Frønsdal, K., Haug, T., *et al*. *In vitro* expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability. *Stem Cells*, 2005;**23**(9):1357–1366.
33 Murphy, J.M., Dixon, K., Beck, S., *et al*. Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum*, 2002;**46**(3):704–713.
34 Solchaga, L.A., Penick, K., Goldberg, V.M., *et al*. Fibroblast growth factor-2 enhances proliferation and delays loss of chondrogenic potential in human adult bone-marrow-derived mesenchymal stem cells. *Tissue Eng Part A*, 2010;**16**(3):1009–1019.
35 Barry, F. and Murphy, M. Mesenchymal stem cells in joint disease and repair. *Nat Rev Rheumatol*, 2013;**9**:584–594.
36 Bieback, K., Hecker, A., Kocaömer, A., *et al*. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. *Stem Cells*, 2009;**27**(9):2331–2341.
37 Jakobsen, R.B., Østrup, E., Zhang, X., *et al*. Analysis of the effects of five factors relevant to *in vitro* chondrogenesis of human mesenchymal stem cells using factorial design and high throughput mRNA-profiling. *PLoS ONE*, 2014;**9**(5):e96615.
38 Bieback, K. Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures. *Transfus Med Hemother*, 2013;**40**(5):326–335.
39 Provot, S. and Schipani, E. Fetal growth plate: a developmental model of cellular adaptation to hypoxia. *Ann N Y Acad Sci*, 2007;**1117**:26–39.
40 Provot, S., Zinyk, D., Gunes, Y., *et al*. Hif-1α regulates differentiation of limb bud mesenchyme and joint development. *J Cell Biol*, 2007;**177**(3):451–464.
41 Demoor, M., Ollitrault, D., Gomez-Leduc, T., *et al*. Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochim Biophys Acta*, 2014;**1840**(8):2414–2440.
42 Leijten, J., Georgi, N., Moreira Teixeira, L., *et al*. Metabolic programming of mesenchymal stromal cells by oxygen tension directs chondrogenic cell fate. *Proc Natl Acad Sci U S A*, 2014;**111**(38):13954–13959.
43 Mathieu, P.S. and Loboa, E.G. Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. *Tissue Eng Part B Rev*, 2012;**18**(6):436–444.
44 Pittenger, M.F., Mackay, A.M., Beck, S.C., *et al*. Multilineage potential of adult human mesenchymal stem cells. *Science*, 1999;**284**(5411):143–147.
45 Bhumiratana, S., Eton, R.E., Oungoulian, S.R., *et al*. Large, stratified, and mechanically functional human cartilage grown *in vitro* by mesenchymal condensation. *Proc Natl Acad Sci U S A*, 2014;**111**(19):6940–6945.
46 Qiu, J. Epigenetics: unfinished symphony. *Nature*, 2006;**441**(7090):143–145.
47 Collas, P., Lund, E.G. and Oldenburg, A.R. Closing the (nuclear) envelope on the genome: how nuclear lamins interact with promoters and modulate gene expression. *Bioessays*, 2014;**36**(1):75–83.
48 Zimmermann, P., Boeuf, S., Dickhut, A., *et al*. Correlation of COL10A1 induction during chondrogenesis of mesenchymal stem cells with demethylation of two CpG sites in the COL10A1 promoter. *Arthritis Rheum*, 2008;**58**(9):2743–2753.
49 Fernandes, A.M., Herlofsen, S.R., Karlsen, T.A., *et al*. Similar properties of chondrocytes from osteoarthritis joints and

mesenchymal stem cells from healthy donors for tissue engineering of articular cartilage. *PLoS ONE*, 2013;**8**(5):e62994.
50 Smith, Z.D. and Meissner, A. DNA methylation: roles in mammalian development. *Nat Rev Genet*, 2013;**14**(3):204–220.
51 Ezura, Y., Sekiya, I., Koga, H., *et al.* Methylation status of CpG islands in the promoter regions of signature genes during chondrogenesis of human synovium-derived mesenchymal stem cells. *Arthritis Rheum*, 2009;**60**(5):1416–1426.
52 Herlofsen, S.R., Bryne, J.C., Hølby, T., *et al.* Genome-wide map of quantified epigenetic changes during *in vitro* chondrogenic differentiation of primary human mesenchymal stem cells. *BMC Genomics*, 2013;**14**(1):105.
53 Lindeman, L.C., Andersen, I.S., Reiner A.H., *et al.* Prepatterning of developmental gene expression by modified histones before zygotic genome activation. *Dev Cell*, 2011;**21**(6):993–1004.
54 He, L. and Hannon, G.J. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet* 2004;**5**:522–531. (Erratum: *Nat Rev Genet* 2004; **5**(8): 631.).
55 Gregory, R.I., Chendrimada, T.P., and Shiekhattar, R. MicroRNA biogenesis: isolation and characterization of the microprocessor complex. *Methods Mol Biol*, 2006;**342**:33–47.
56 Bartel, D.P. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell*, 2004;**116**(2):281–297.
57 Lee, R.C., Feinbaum, R.L., and Ambros, V. The *C. elegans* heterochronic gene *lin-4* encodes small RNAs with antisense complementarity to *lin-14*. *Cell*, 1993;**75**(5):843–854.
58 Hutvagner, G. and Zamore, P.D. A microRNA in a multiple-turnover RNAi enzyme complex. *Science*, 2002;**297**(5589):2056–2060.
59 Orom, U.A., Nielsen, F.C., and Lund, A.H. MicroRNA-10a binds the 5′UTR of ribosomal protein mRNAs and enhances their translation. *Mol Cell*, 2008;**30**(4):460–471.
60 Place, R.F., Li, L.C., Pookot, D., *et al.* MicroRNA-373 induces expression of genes with complementary promoter sequences. *Proc Natl Acad Sci U S A*, 2008;**105**(5):1608–1613.
61 Tay, Y., Zhang, J., Thomson, A.M., *et al.* MicroRNAs to *Nanog, Oct4* and *Sox2* coding regions modulate embryonic stem cell differentiation. *Nature*, 2008;**455**(7216):1124–1128.
62 Lin, C.C., Liu, L.Z., Addison, J.B., *et al.* A KLF4–miRNA-206 autoregulatory feedback loop can promote or inhibit protein translation depending upon cell context. *Mol Cell Biol*, 2011;**31**(12):2513–2527.
63 Vasudevan, S. Posttranscriptional upregulation by microRNAs. *Wiley Interdiscip Rev RNA*, 2012;**3**(3):311–330.
64 Zardo, G., Ciolfi, A., Vian, L., *et al.* Transcriptional targeting by microRNA–polycomb complexes: a novel route in cell fate determination. *Cell Cycle*, 2012;**11**(19):3543–3549.
65 Brennecke, J., Stark, A., Russell, R.B., and Cohen, S.M. Principles of microRNA-target recognition. *PLoS Biol*, 2005;**3**(3):e85.
66 Selbach, M., Schwanhäusser, B., Thierfelder, N., *et al.* Widespread changes in protein synthesis induced by microRNAs. *Nature*, 2008;**455**(7209):58–63.
67 Karlsen, T.A., Jakobsen, R.B., Mikkelsen, T.S., and Brinchmann, J.E. microRNA-140 targets *RALA* and regulates chondrogenic differentiation of human mesenchymal stem cells by translational enhancement of *SOX9* and *ACAN*. *Stem Cells Dev*, 2014;**23**(3):290–304.
68 Friedman, R.C., Farh, K.K., Burge, C.B., and Bartel, D.P. Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res*, 2009;**19**(1):92–105.
69 Kobayashi, T., Lu, J., Cobb, B.S., *et al.* Dicer-dependent pathways regulate chondrocyte proliferation and differentiation. *Proc Natl Acad Sci U S A*, 2008;**105**(6):1949–1954.
70 Wienholds, E., Kloosterman, W.P., Miska, E., *et al.* MicroRNA expression in zebrafish embryonic development. *Science*, 2005;**309**(5732):310–311.
71 Tuddenham, L., Wheeler, G., Ntounia-Fousara, S., *et al.* The cartilage specific microRNA-140 targets histone deacetylase 4 in mouse cells. *FEBS Lett*, 2006;**580**(17):4214–4217.
72 Yang, J., Qin, S., Yi, C., *et al.* MiR-140 is co-expressed with *Wwp2-C* transcript and activated by *Sox9* to target *Sp1* in maintaining the chondrocyte proliferation. *FEBS Lett*, 2011;**585**(19):2992–2997.
73 Yamashita, S., Miyaki, S., Kato, Y., *et al.* L-Sox5 and Sox6 proteins enhance chondrogenic miR-140 microRNA expression by strengthening dimeric Sox9 activity. *J Biol Chem*, 2012;**287**(26):22206–22215.
74 Miyaki, S., Sato, T., Inoue, A., *et al.* MicroRNA-140 plays dual roles in both cartilage development and homeostasis. *Genes Dev*, 2010;**24**(11):1173–1185.
75 Deniaud, E., Baguet, J., Chalard, R., *et al.* Overexpression of transcription factor Sp1 leads to gene expression perturbations and cell cycle inhibition. *PLoS ONE*, 2009;**4**(9):e7035.
76 Nakamura, Y., Inloes, J.B., Katagiri, T., and Kobayashi, T. Chondrocyte-specific microRNA-140 regulates endochondral bone development and targets *Dnpep* to modulate bone morphogenetic protein signaling. *Mol Cell Biol*, 2011;**31**(14):3019–3028.
77 Eberhart, J.K., He, X., Swartz, M.E., *et al.* MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet*, 2008;**40**(3):290–298.
78 Pais, H., Nicolas, F.E., Soond, S.M., *et al.* Analyzing mRNA expression identifies Smad3 as a microRNA-140 target regulated only at protein level. *RNA*, 2010;**16**(3):489–494.
79 Nicolas, F.E., Pais, H., Schwach, F., *et al.* Experimental identification of microRNA-140 targets by silencing and overexpressing miR-140. *RNA*, 2008;**14**(12):2513–2520.
80 Nicolas, F.E., Pais, H., Schwach, F., *et al.* mRNA expression profiling reveals conserved and non-conserved miR-140 targets. *RNA Biol*, 2011;**8**(4):607–615.
81 Canalis, E., Adams, D.J., Boskey, A., *et al.* Notch signaling in osteocytes differentially regulates cancellous and cortical bone remodeling. *J Biol Chem*, 2013;**288**(35):25614–25625.
82 Duprez, D., Bell, E.J., Richardson, M.K., *et al.* Overexpression of BMP-2 and BMP-4 alters the size and shape of developing skeletal elements in the chick limb. *Mech Dev*, 1996;**57**(2):145–157.
83 Monsoro-Burq, A.H., Duprez, D,. Watanabe, Y., *et al.* The role of bone morphogenetic proteins in vertebral development. *Development*, 1996;**122**(11):3607–3616.
84 Son, B.R., Marquez-Curtis, L.A., Kucia, M., *et al.* Migration of bone marrow and cord blood mesenchymal stem cells in vitro is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells*, 2006;**24**(5):1254–1264.
85 Murata, K., Kitaori, T., Oishi, S., *et al.* Stromal cell-derived factor 1 regulates the actin organization of chondrocytes and chondrocyte hypertrophy. *PLoS ONE*, 2012;**7**(5):e37163.
86 Lisignoli, G., Toneguzzi, S., Piacentini, A., *et al.* CXCL12 (SDF-1) and CXCL13 (BCA-1) chemokines significantly induce

proliferation and collagen type I expression in osteoblasts from osteoarthritis patients. *J Cell Physiol*, 2006;**206**(1):78–85.

87 Xu, Q., Sun, X.C., Shang, X.P., and Jiang, H.S. Association of CXCL12 levels in synovial fluid with the radiographic severity of knee osteoarthritis. *J Investig Med*, 2012;**60**(6):898–901.

88 Herlofsen, S.R., Küchler, A.M., Melvik, J.E., and Brinchmann, J.E. Chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in self-gelling alginate discs reveals novel chondrogenic signature gene clusters. *Tissue Eng Part A*, 2011;**17** (7–8):1003–1013.

89 Kim, I.S., Otto, F., Zabel, B., and Mundlos, S. Regulation of chondrocyte differentiation by *Cbfa1*. *Mech Dev*, 1999;**80**(2):159–170.

90 Karlsen, T.A., Shahdadfar, A., and Brinchmann, J.E. Human primary articular chondrocytes, chondroblasts-like cells, and dedifferentiated chondrocytes: differences in gene, microRNA, and protein expression and phenotype. *Tissue Eng Part C Methods*, 2011;**17**(2):219–227.

91 Nakamura, Y., He, X., Kato, H., *et al*. *Sox9* is upstream of microRNA-140 in cartilage. *Appl Biochem Biotechnol*, 2012;**166** (1):64–71.

92 Iliopoulos, D., Malizos, K.N., Oikonomou, P., and Tsezou, A. Integrative microRNA and proteomic approaches identify novel osteoarthritis genes and their collaborative metabolic and inflammatory networks. *PLoS ONE*, 2008;**3**(11):e3740.

93 Patel, V. and Noureddine, L. MicroRNAs and fibrosis. *Curr Opin Nephrol Hypertens*, 2012;**21**(4):410–416.

94 Knutsen, G., Drogset, J.O., Engebretsen, L., *et al*. A randomized trial comparing autologous chondrocyte implantation with microfracture. Findings at five years. *J Bone Joint Surg Am*, 2007;**89** (10):2105–2112.

95 Zhang, Y., Jia, J., Yang, S., *et al*. MicroRNA-21 controls the development of osteoarthritis by targeting GDF-5 in chondrocytes. *Exp Mol Med*, 2014;**46**:e79.

96 Ham, O., Lee, C.Y., Song, B., *et al*. Upregulation of miR-23b enhances the autologous therapeutic potential for degenerative arthritis by targeting PRKACB in synovial fluid-derived mesenchymal stem cells from patients. *Mol Cells*, 2014;**37**(6):449–456.

97 Sun, J., Zhong, N., Li, Q., *et al*. MicroRNAs of rat articular cartilage at different developmental stages identified by Solexa sequencing. *Osteoarthritis Cartilage*, 2011;**19**(10):1237–1245.

98 Suomi, S., Taipaleenmäki, H., Seppänen, A., *et al*. MicroRNAs regulate osteogenesis and chondrogenesis of mouse bone marrow stromal cells. *Gene Regul Syst Bio*, 2008;**2**:177–191.

99 Yang, B., Guo, H., Zhang, Y., *et al*. The microRNA expression profiles of mouse mesenchymal stem cell during chondrogenic differentiation. *BMB Rep*, 2011;**44**(1):28–33.

100 Gradus, B., Alon, I., and Hornstein, E. miRNAs control tracheal chondrocyte differentiation. *Dev Biol*, 2011;**360**(1):58–65.

101 Vonk, L.A., Kragten, A.H., Dhert, W.J., *et al*. Overexpression of hsa-miR-148a promotes cartilage production and inhibits cartilage degradation by osteoarthritic chondrocytes. *Osteoarthritis Cartilage*, 2014;**22**(1):145–153.

102 Bhushan, R., Grünhagen, J., Becker, J., *et al*. miR-181a promotes osteoblastic differentiation through repression of TGF-β signaling molecules. *Int J Biochem Cell Biol*, 2012;**45**(3):696–705.

103 Sumiyoshi, K., Kubota, S., Ohgawara, T., *et al*. Novel role of miR-181a in cartilage metabolism. *J Cell Biochem*, 2013;**114** (9):2094–2100.

104 Chan, Y.C., Banerjee, J., Choi, S.Y., and Sen, C.K. miR-210: the master hypoxamir. *Microcirculation*, 2012;**19**(3):215–223.

105 Kawanishi, Y., Nakasa, T., Shoji, T., *et al*. Intra-articular injection of synthetic microRNA-210 accelerates avascular meniscal healing in rat medial meniscal injured model. *Arthritis Res Ther*, 2014;**16** (6):488.

106 Zhang, Z., Kang, Y., Zhang, Z., *et al*. Expression of microRNAs during chondrogenesis of human adipose-derived stem cells. *Osteoarthritis Cartilage*, 2012;**20**(12):1638–1646.

107 Dunn, W., DuRaine, G., and Reddi, A.H. Profiling microRNA expression in bovine articular cartilage and implications for mechanotransduction. *Arthritis Rheum*, 2009;**60**(8):2333–2339.

108 Swingler, T.E., Wheeler, G., Carmont, V., *et al*. The expression and function of microRNAs in chondrogenesis and osteoarthritis. *Arthritis Rheum*, 2012;**64**(6):1909–1919.

109 Bakhshandeh, B., Soleimani, M., Paylakhi, S.H., and Ghaemi, N. A microRNA signature associated with chondrogenic lineage commitment. *J Genet*, 2012;**91**(2):171–182.

110 Lolli, A., Lambertini, E., Penolazzi, L., *et al*. Pro-chondrogenic effect of miR-221 and slug depletion in human MSCs. *Stem Cell Rev*, 2014;**10**(6):841–855.

111 Kim, D., Song, J., and Jin, E.J. MicroRNA-221 regulates chondrogenic differentiation through promoting proteosomal degradation of slug by targeting Mdm2. *J Biol Chem*, 2010;**285**(35): 26900–26907.

112 Tome, M., López-Romero, P., Albo, C., *et al*. miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. *Cell Death Differ*, 2011;**18**(6):985–995.

113 Martinez-Sanchez, A., Dudek, K.A., and Murphy, C.L. Regulation of human chondrocyte function through direct inhibition of cartilage master regulator SOX9 by microRNA-145 (miRNA-145). *J Biol Chem*, 2012;**287**(2):916–924.

114 Yang, B., Guo, H., Zhang, Y., *et al*. MicroRNA-145 regulates chondrogenic differentiation of mesenchymal stem cells by targeting *Sox9*. *PLoS ONE*, 2011;**6**(7):e21679.

115 Dudek, K.A., Lafont, J.E., Martinez-Sanchez, A., and Murphy, C.L. Type II collagen expression is regulated by tissue-specific miR-675 in human articular chondrocytes. *J Biol Chem*, 2010;**285**(32): 24381–24387.

116 Lin, E.A., Kong, L., Bai, X.H., *et al*. miR-199a, a bone morphogenic protein 2-responsive MicroRNA, regulates chondrogenesis via direct targeting to Smad1. *J Biol Chem*, 2009;**284**(17):11326–11335.

117 Sumiyoshi, K., Kubota, S., Ohgawara, T., *et al*. Identification of miR-1 as a micro RNA that supports late-stage differentiation of growth cartilage cells. *Biochem Biophys Res Commun*, 2010; **402**(2):286–290.

118 Guan, Y.J., Yang, X., Wei, L., and Chen, Q. MiR-365: a mechanosensitive microRNA stimulates chondrocyte differentiation through targeting histone deacetylase 4. *FASEB J*, 2011;**25** (12):4457–4466.

119 McAlinden, A., Varghese, N., Wirthlin, L., and Chang, L. Differentially expressed microRNAs in chondrocytes from distinct regions of developing human cartilage. *PLoS ONE*, 2013;**8**(9): e75012.

120 Eskildsen, T., Taipaleenmäki, H., Stenvang, J., *et al*. MicroRNA-138 regulates osteogenic differentiation of human stromal (mesenchymal) stem cells in vivo. *Proc Natl Acad Sci U S A*, 2011;**108**(15): 6139–6144.

121 Yang, Z., Bian, C., Zhou, H., *et al*. MicroRNA hsa-miR-138 inhibits adipogenic differentiation of human adipose tissue-derived mesenchymal stem cells through adenovirus EID-1. *Stem Cells Dev*, 2011;**20**(2):259–267.

122 Lee, S., Yoon, D.S., Paik, S., *et al*. microRNA-495 inhibits chondrogenic differentiation in human mesenchymal stem cells by targeting Sox9. *Stem Cells Dev*, 2014;**23**(15):1798–1808.

123 Canty, E.G. and Kadler, K.E. Procollagen trafficking, processing and fibrillogenesis. *J Cell Sci*, 2005;**118**(Pt 7):1341–1353.

124 Olderøy, M.Ø., Lilledahl, M.B., Beckwith, M.S., *et al*. Biochemical and structural characterization of neocartilage formed by mesenchymal stem cells in alginate hydrogels. *PLoS ONE*, 2014;**9**(3): e91662.

125 Ikeda, T., Kamekura, S., Mabuchi, A., *et al*. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. *Arthritis Rheum*, 2004; **50**(11):3561–3573.

126 Herlofsen, S.R., Høiby, T., Cacchiarelli, D., *et al*. Importance of SOX8 for *in vitro* chondrogenic differentiation of human mesenchymal stromal cells. *Stem Cells*, 2014;**32**(6):1629–1635.

127 Kadler, K.E., Hill, A., and Canty-Laird, E.G. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. *Curr Opin Cell Biol*, 2008;**20**(5):495–501.

128 Sekiya, I., Larson, B.L., Vuoristo, J.T., *et al*. Comparison of effect of BMP-2, -4, and -6 on *in vitro* cartilage formation of human adult stem cells from bone marrow stroma. *Cell Tissue Res*, 2005; **320**(2):269–276.

129 Handorf, A.M. and Li, W.J. Fibroblast growth factor-2 primes human mesenchymal stem cells for enhanced chondrogenesis. *PLoS ONE*, 2011;**6**(7):e22887.

130 Dombrowski, C., Helledie, T., Ling, L., *et al*. FGFR1 signaling stimulates proliferation of human mesenchymal stem cells by inhibiting the cyclin-dependent kinase inhibitors $p21^{Waf1}$ and $p27^{Kip1}$. *Stem Cells*, 2013;**31**(12):2724–2736.

131 Johnstone, B., Hering, T.M., Caplan, A.I., *et al*. *In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res*, 1998;**238**(1):265–272.

132 Zimmerman, L.B., De Jesús-Escobar, J.M., and Harland, R.M. The Spemann organizer signal noggin binds and inactivates bone morphogenetic protein 4. *Cell*, 1996;**86**(4):599–606.

133 Oldershaw, R.A., Tew, S.R., Russell, A.M., *et al*. Notch signaling through Jagged-1 is necessary to initiate chondrogenesis in human bone marrow stromal cells but must be switched off to complete chondrogenesis. *Stem Cells*, 2008;**26**(3):666–674.

CHAPTER 15

# The role of the extracellular matrix in the differentiation of mesenchymal stromal cells

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## 15.1 Summary

Mesenchymal stromal cells (MSCs) are a heterogeneous population of stromal cells that exist in almost all tissues. Owing to their relatively easy acquisition, large-scale expansion capability, and their ability to differentiate into cells of mesenchymal lineages, MSCs are a promising candidate for cell-based tissue regeneration. The regulation of their lineage commitment, especially that of adipogenic and osteogenic differentiation, has been widely studied. The microenvironment of the MSC niche, including the geometric extracellular matrix (ECM), adjacent cells, and a number of cytokines and growth factors, are critical for their lineage commitment. ECM, as a major component of the MSC niche microenvironment, is essential for cell fate determination by providing specific geometric cues and direct interactions with cell surface receptors. This chapter reviews advances in elucidating the role of the ECM in regulating MSC differentiation.

## 15.2 Multipotency of mesenchymal stromal cells

Friedenstein *et al.*, who discovered MSCs, implanted them under the renal capsule or in diffusion chambers and then harvested the implants for histological examination 30–90 days later. They observed bone formation and hematopoiesis in the MSC implants [1]. In 1999, Pittenger *et al.* induced MSCs to differentiate *in vitro* into osteoblasts, adipocytes, and chondrocytes by using various combinations of chemicals [2]. These pioneering studies clearly demonstrated the multipotency of MSCs in the mesenchymal lineage. This ability of MSCs to differentiate into cells of the mesenchymal lineage has been recognized as one of the criteria by which MSCs are defined [3]. The differentiation ability of MSCs has been extensively studied, mainly using well-established *in vitro* assays with clonally expanded MSCs. Typically, MSCs cultured in medium with 1-methyl-3-isobutylxanthine (IBMX), dexamethasone, insulin, and indomethacin differentiate into adipocytes [4]. IBMX and dexamethasone are important for the initiation of adipogenic differentiation. IBMX is believed to inhibit phosphodiesterases, which cause an elevation of intracellular cyclic adenosine monophosphate [5]. Elevation of cyclic adenosine monophosphate leads to alteration of transcription factors through the protein kinase A pathway. IBMX has also been shown to directly induce

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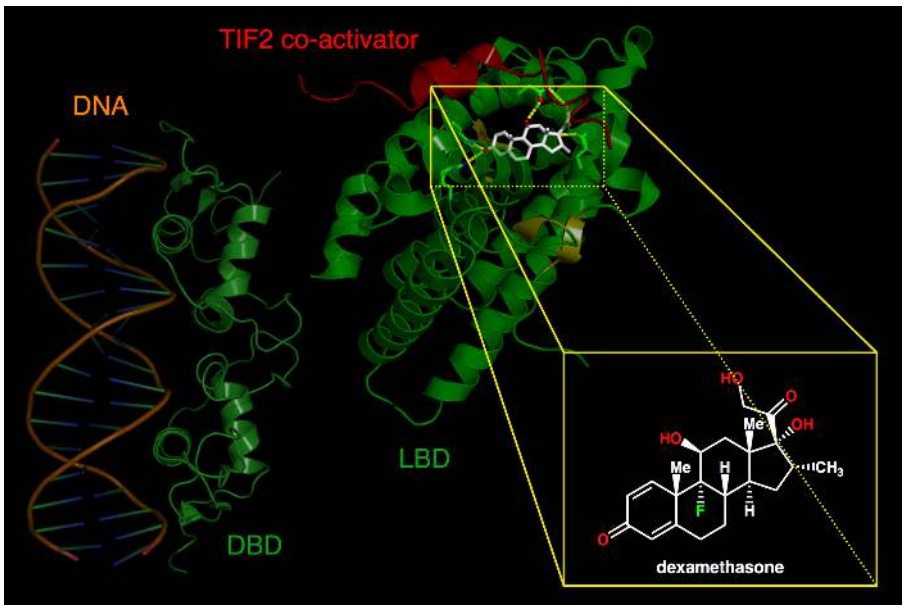



**Figure 15.1** Crystallographic structures of the glucocorticoid receptor DNA binding domain (DBD, left, PDB: 1R4O bound to DNA) and ligand binding domain (LBD, right, 1M2Z bound to dexamethasone and the TIF2 co-activator protein). Dashed lines represent hydrogen bonding interactions between the receptor and ligand. The two-dimensional (2D) structure of dexamethasone is also depicted in the lower right-hand side of the picture for reference. *Source*: https://commons.wikimedia.org/wiki/File:Glucocorticoid_receptor.png#/media/File:Glucocorticoid_receptor.png.

CCAAT-enhancer-binding protein (C/EBP)-β expression [6]. Dexamethasone activates C/EBPδ expression through binding to the glucocorticoid receptor [6] (Figure 15.1).

Indomethacin is a well-known inhibitor of cyclooxygenase 1/2 (Cox1/2) activities. However, the adipogenic activity of indomethacin is not related to the inhibition of Cox1/2 but rather by activation of peroxisome proliferator-activated receptor-γ (PPAR-γ) [7]. Insulin functions as a promoter of glucose uptake, which is stored as triglycerides in adipocytes.

For *in vitro* osteogenic differentiation MSCs are normally cultured with β-glycerophosphate sodium (β-GP), L-ascorbic acid (vitamin C), and dexamethasone [8]. To analyze the effects of these chemicals on the differentiation of MSCs, Coelho and Fernandes cultured MSCs in standard medium supplemented with vitamin C, β-GP, or dexamethasone alone or in the combinations vitamin C + β-GP or vitamin C + β-GP + dexamethasone [9]. Vitamin C was found to initiate the formation of a collagenous extracellular matrix that led to upregulation of alkaline phosphatase (ALP) and osteocalcin [10]. Dexamethasone promoted cell proliferation, resulting in an increase in ALP activity and mineral deposition. β-GP could be hydrolyzed by ALP, which provided a high level of phosphate ions for mineral deposition of ECM [9].

However, *in vivo*, MSC fate decision is much more complex and plastic [11]. In healthy individuals under normal conditions there is a reciprocal relationship between adipocyte and osteoblast differentiation of MSCs. Alteration in this balance of MSC differentiation is believed to occur in a number of diseases, most notably osteoporosis [12]. Conversely, various diseases, particularly obesity, alter the differentiation potential of MSCs [13,14]. Interestingly, it has been shown that adipogenic differentiation was favored in MSCs derived from people with osteoporosis or aging people. Corroborating these findings, numerous *in vitro* studies have demonstrated that fat-induction factors can inhibit osteogenesis [15,16] and that bone-induction factors can inhibit adipogenesis [17]. Thus, there is evidence of a reciprocal relationship between adipogenic and osteogenic differentiation in MSCs.

## 15.3 The extracellular matrix and mesenchymal stromal cell differentiation

Stromal cell differentiation is also mediated in part by signals from the extracellular environment. Although many reports have shown that cytokines and growth factors are the primary determinants of stromal cell lineage differentiation, much attention has also focused on molecules within the ECM, as well as those from neighboring cells during MSC fate determination [18]. The ECM is an acellular component secreted by cells present in all tissues and organs. It provides not only critical physical scaffolding for tissue remodeling, but also initiates important biological cues that control cell differentiation, adhesion, and interaction with other cells [19]. ECM components, such as osteopontin (OPN), cadherins, ephrins, and Notch family members, have been reported to regulate cell differentiation [20–22].

Cell fate regulation by the ECM can be exerted via two main mechanisms: (1) the harboring of growth factors or growth-factor-binding proteins and (2) direct regulation of cell behavior either through receptor-mediated signaling or by modulating the cellular response to growth factors. Interestingly, ECM components have also been found to play an active role in regulating the mobilization of growth factors and cytokines rather than passively impeding their binding to their corresponding receptors. Mutation of the ECM remodeling enzyme matrix

metalloproteinase (MMP) 9 resulted in delayed bone development as well as altered vascular differentiation and tissue repair ability [23]. Ligation of cell surface integrins by ECM components can directly induce downstream signaling pathways, the so-called outside–in signaling. There are 24 distinct integrins assembled from 8 β and 18 α subunits [24]. The diversity of ECM receptors and their ligands allows MSCs to monitor their own microenvironment and respond to various kinds of stimulation. Thus, the complex signaling network downstream of these receptors provides a source of diversity for cell–matrix interactions. These two types of regulatory mechanisms do not act in isolation from each other; rather, they crosstalk to each other. In the following we discuss several key regulators, including cytokines and geometric cues, to demonstrate the critical roles of the ECM in directing MSC differentiation.

### 15.3.1 The role of osteopontin in mesenchymal stromal cell differentiation

OPN, also known as secreted phosphoprotein 1, is a major component of noncollagenous ECM. Extracellular OPN exerts its effects through interactions with multiple ubiquitously expressed cell surface receptors, including integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$, $\alpha_4\beta_1$, $\alpha_8\beta_1$, and $\alpha_9\beta_1$) [25–27] and the non-integrin receptor CD44, which is the receptor for hyaluronic acid [28,29]. Upon ligation of OPN receptors different cell signals are generated and various tasks are performed, including cell adhesion [30], cell survival [31], and cell migration [32]. OPN is expressed by a variety of cell types, including fibroblasts, osteoblasts, and a range of immune cells [33]. It is deposited in the ECM and is present in circulating fluids. Moreover, OPN is also expressed during numerous physiological and pathological processes. Because of its widespread expression it has been implicated in cancer metastasis, progression of autoimmune disease, bone remodeling, wound repair, and responses to stress [33–35].

Bone has been implicated as an endocrine organ in energy metabolism by exerting effects through molecules secreted by osteoblasts and osteoclasts [36]. OPN is an osteoblast biomarker and is abundantly secreted by MSCs. It is upregulated during osteogenesis. However, its role in MSC differentiation has not been elucidated. Compared with wild-type MSCs, our group found that adipogenic differentiation of $OPN^{-/-}$ MSCs was dramatically accelerated while osteogenic differentiation was inhibited. Consistent with these findings, specific knockdown of OPN by shRNA promoted robust adipogenic differentiation and inhibited osteogenic differentiation. Re-expression of OPN restored a normal balance between adipogenesis and osteogenesis in $OPN^{-/-}$ MSCs. Retardation of bone formation by $OPN^{-/-}$ MSCs was verified by *in vivo* implantation of MSCs with hydroxyapatite–tricalcium phosphate, a bone-forming matrix. The role of extracellular OPN in MSC differentiation was also demonstrated by supplementation and neutralization of OPN. Blocking the highly expressed OPN receptors integrin $\alpha_v/\beta_1$, but not CD44, also affected the balance of MSC differentiation between adipocytes and osteoblasts. Further studies revealed that OPN inhibits the signaling pathway of C/EBPs through integrin $\alpha_v/\beta_1$ receptors. Consistent with these *in vitro* results, $OPN^{-/-}$ mice had a higher fat to total body weight ratio than wild-type mice. Thus, the interaction of OPN and integrin $\alpha_v/\beta_1$ plays a key role in regulating MSC differentiation.

### 15.3.2 Geometric cues in mesenchymal stromal cell differentiation

Nanoscale symmetry and disorder have been shown to play important roles in dictating various types of cell behavior and stromal cell differentiation [37,38]. Geometric cues, such as the stiffness and topology (biophysical forces) of the cell microenvironment, which are dictated by the ECM *in vivo*, have dramatic effects on MSC lineage commitment [39]. By culturing geometrically patterned MSCs in medium containing either adipogenic or osteogenic chemical inducers, Kilian *et al.* showed that geometric cues suitable for the contractile cytoskeleton were osteogenic, while those disrupting contractility were adipogenic [40].

Micropatterning technology, which is able to precisely control cell attachment by depositing specific proteins on a substrate, has been widely used to geometrically define the substrate surface in order to study the interaction between MSCs and patterned substrates [41]. Nanoscale cues have been reported to induce a change in cell shape by altering the organization and assembly of the cytoskeleton. It has been shown that the balance between adipogenic and osteogenic differentiation of MSCs was disrupted by micropatterned protein-induced cell shape change [42]. Although the Rho/ROCK (Ras homolog/Rho-associated protein kinase) pathway has been implicated during this process, details of the mechanisms involved in geometric cue-induced MSC differentiation are still largely elusive.

To better mimic the microenvironment of stromal cell differentiation *in vivo*, powerful three-dimensional (3D) culture systems have been developed (see Chapter 8). The 3D culture system allows us to artificially control cell shape more precisely compared with the use of cell monolayers [43]. The 3D systems most commonly used involve poly(ethylene glycol)-based hydrogels. In such systems, cells are more rounded and under spatial orientation similar to that found with the ECM *in vivo*, unlike cells cultured in standard 2D systems. It has been reported that the shape of MSCs can be modulated dynamically by creating photodegradable poly(ethylene glycol)-based hydrogels [44]. This system makes it possible to study complex physical interactions between MSCs and the ECM. Thus, the 3D culture system provides a much improved opportunity by which to study the detailed mechanism of lineage commitment of MSCs *in vivo*.

### 15.3.3 Crosstalk between the extracellular matrix and mesenchymal stromal cells

In addition to their passive response to ECMs, MSCs also possess the ability to actively remodel their ECM microenvironment. A study by Tang *et al.* showed that MSCs remodeled the ECM by secreting a membrane-anchored metalloproteinase MT1-MMP

(MMP 14) [45]. Since deletion of MT1-MMP is embryonic lethal, the authors generated a conditional knockout mouse in which MT1-MMP was specifically deleted in mesenchymal progenitors and skeletal stromal cells. These mice showed a decrease in bone formation and an increase in bone marrow adipose cells and articular cartilage generation. Interestingly, MSCs isolated from these MT1-MMP conditional knockout mice were able to differentiate normally into osteoblasts in a traditional 2D culture system. However, when cultured in 3D collagen gel, these MSCs tended to differentiate into adipocytes rather than osteoblasts. Further study showed that MT1-MMP exerted its effect on MSC lineage commitment through remodeling the ECM. This, in turn, altered the cell shape and ultimately activated the downstream cell signaling cascade involved during MSC differentiation.

## 15.4 Conclusions and future perspectives

In today's society obesity, diabetes mellitus, and osteoporosis are major public health concerns. Investigation of the relationship between bone and fat in energy metabolism are being explored, aiming at improving human health and reducing the burden on society. Osteoblasts and adipocytes are both derived from MSCs and constitute the major cellular components of bone and fat respectively. Ever since Meunier *et al.* reported the replacement of the cell populations in the marrow by adipose tissue in people with osteoporosis [46], the intimately competitive balance between adipocytes and osteoblasts in bone marrow has attracted significant attention.

A series of important factors have been implicated in the fate decision of MSCs. Among these, the cues and signals originating from the ECM play a critical role in regulating MSC lineage commitment *in vivo*. In addition, previous studies on the role of the ECM in MSC differentiation provide critical insight into the superiority of 3D system in modeling the *in vivo* situation *in vitro*. The widely used 2D culture systems are inadequate for addressing questions about the microenvironment *in vivo*, where stromal cells undergo lineage commitment. In conclusion, this chapter has discussed how ECM and the cytokines and growth factors in the microenvironment exert critical roles during the lineage commitment of MSCs.

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## References

1 A. J. Friedenstein, I. I. Piatetzky-Shapiro, K. V. Petrakova. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;**16**(3):381–390.

2 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411): 143–147.

3 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006; **8**(4):315–317.

4 M. A. Scott, V. T. Nguyen, B. Levi, A. W. James. Current methods of adipogenic differentiation of mesenchymal stem cells. *Stem Cells Dev* 2011;**20**(10):1793–1804.

5 P. K. Brindle, M. R. Montminy. The CREB family of transcription activators. *Curr Opin Genet Dev* 1992;**2**(2):199–204.

6 Z. Cao, R. M. Umek, S. L. McKnight. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev* 1991;**5**(9):1538–1552.

7 J. M. Lehmann, J. M. Lenhard, B. B. Oliver, G. M. Ringold, S. A. Kliewer. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other non-steroidal anti-inflammatory drugs. *J Biol Chem* 1997;**272**(6):3406–3410.

8 H. Hosseinkhani, M. Hosseinkhani, F. Tian, H. Kobayashi, Y. Tabata. Osteogenic differentiation of mesenchymal stem cells in self-assembled peptide-amphiphile nanofibers. *Biomaterials* 2006;**27**(22):4079–4086.

9 M. J. Coelho, M. H. Fernandes. Human bone cell cultures in biocompatibility testing. Part II: effect of ascorbic acid, β-glycerophosphate and dexamethasone on osteoblastic differentiation. *Biomaterials* 2000;**21**(11):1095–1102.

10 R. T. Franceschi, B. S. Iyer, Y. Cui. Effects of ascorbic acid on collagen matrix formation and osteoblast differentiation in murine MC3T3-E1 cells. *J Bone Miner Res* 1994;**9**(6):843–854.

11 J. M. Gimble. The function of adipocytes in the bone marrow stroma. *New Biol* 1990;**2**(4):304–312.

12 J. P. Rodriguez, P. Astudillo, S. Ríos, A. M. Pino. Involvement of adipogenic potential of human bone marrow mesenchymal stem cells (MSCs) in osteoporosis. *Curr Stem Cell Res Ther* 2008;**3**(3):208–218.

13 C. L. Wu, B. O. Diekman, D. Jain, F. Guilak. Diet-induced obesity alters the differentiation potential of stem cells isolated from bone marrow, adipose tissue and infrapatellar fat pad: the effects of free fatty acids. *Int J Obes (Lond)* 2013;**37**(8):1079–1087.

14 B. Onate, G. Vilahur, S. Camino-López, *et al.* Stem cells isolated from adipose tissue of obese patients show changes in their transcriptomic profile that indicate loss in stemcellness and increased commitment to an adipocyte-like phenotype. *BMC Genomics* 2013;**14**:625.

15 E. Solheim, E. M. Pinholt, G. Bang, E. Sudmann. Inhibition of heterotopic osteogenesis in rats by a new bioerodible system for local delivery of indomethacin. *J Bone Joint Surg Am* 1992; **74**(5):705–712.

16 D. C. Yang, H. J. Tsay, S. Y. Lin, *et al.* cAMP/PKA regulates osteogenesis, adipogenesis and ratio of RANKL/OPG mRNA expression in mesenchymal stem cells by suppressing leptin. *PLoS ONE* 2008;**3**(2):e1540.

17 F. Rahman, F. Al Frouh, B. Bordignon, *et al.* Ascorbic acid is a dose-dependent inhibitor of adipocyte differentiation, probably by reducing cAMP pool. *Front Cell Dev Biol* 2014;**2**:29.

18 C. Streuli. Extracellular matrix remodelling and cellular differentiation. *Curr Opin Cell Biol* 1999;**11**(5):634–640.

19 C. Frantz, K. M. Stewart, V. M. Weaver. The extracellular matrix at a glance. *J Cell Sci* 2010;**123**(Pt 24):4195–4200.

20 Q. Chen, P. Shou, L. Zhang, *et al*. An osteopontin–integrin interaction plays a critical role in directing adipogenesis and osteogenesis by mesenchymal stem cells. *Stem Cells* 2014;**32**(2):327–337.

21 K. J. Hodivala, F. M. Watt. Evidence that cadherins play a role in the downregulation of integrin expression that occurs during keratinocyte terminal differentiation. *J Cell Biol* 1994;**124**(4):589–600.

22 S. Lin, B. Wang, S. Getsios. Eph/ephrin signaling in epidermal differentiation and disease. *Semin Cell Dev Biol* 2012;**23**(1):92–101.

23 T. H. Vu, J. M. Shipley, G. Bergers, *et al*. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998;**93**(3):411–422.

24 R. O. Hynes. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;**110**(6):673–687.

25 A. O'Regan, J. S. Berman. Osteopontin: a key cytokine in cell-mediated and granulomatous inflammation. *Int J Exp Pathol* 2000;**81**(6):373–390.

26 Y. Yokosaki, K. Tanaka, F. Higashikawa, K. Yamashita, A. Eboshida. Distinct structural requirements for binding of the integrins $\alpha_v\beta_6$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ and $\alpha_9\beta_1$ to osteopontin. *Matrix Biol* 2005; **24**(6):418–427.

27 S. T. Barry, S. B. Ludbrook, E. Murrison, *et al*. Analysis of the $\alpha_4\beta_1$ integrin–osteopontin interaction. *Exp Cell Res* 2000;**258**(2): 342–351.

28 D. T. Denhardt, X. Guo. Osteopontin: a protein with diverse functions. *FASEB J* 1993;**7**(15):1475–1482.

29 G. F. Weber, S. Ashkar, M. J. Glimcher, H. Cantor. Receptor–ligand interaction between CD44 and osteopontin (Eta-1). *Science* 1996; **271**(5248):509–512.

30 S. R. Rittling, A. F. Chambers. Role of osteopontin in tumour progression. *Br J Cancer* 2004;**90**(10):1877–1881.

31 O. Helluin, C. Chan, G. Vilaire, *et al*. The activation state of $\alpha_v\beta_3$ regulates platelet and lymphocyte adhesion to intact and thrombin-cleaved osteopontin. *J Biol Chem* 2000;**275**(24):18337–18343.

32 J. Rice, D. L. Courter, C. M. Giachelli, M. Scatena. Molecular mediators of $\alpha_v\beta_3$-induced endothelial cell survival. *J Vasc Res* 2006;**43**(5):422–436.

33 D. T. Denhardt, M. Noda. Osteopontin expression and function: role in bone remodeling. *J Cell Biochem Suppl* 1998;**30–31**:92–102.

34 K. X. Wang, Y. Shi, D. T. Denhardt. Osteopontin regulates hindlimb-unloading-induced lymphoid organ atrophy and weight loss by modulating corticosteroid production. *Proc Natl Acad Sci U S A* 2007;**104**(37):14777–14782.

35 K. X. Wang, Y. F. Shi, Y. Ron, C. C. Kazanecki, D. T. Denhardt. Plasma osteopontin modulates chronic restraint stress-induced thymus atrophy by regulating stress hormones: inhibition by an anti-osteopontin monoclonal antibody. *J Immunol* 2009;**182**(4): 2485–2491.

36 N. K. Lee, H. Sowa, E. Hinoi, *et al*. Endocrine regulation of energy metabolism by the skeleton. *Cell* 2007;**130**(3):456–469.

37 J. Huang, S. V. Grater, F. Corbellini, *et al*. Impact of order and disorder in RGD nanopatterns on cell adhesion. *Nano Lett* 2009;**9**(3): 1111–1116.

38 R. J. McMurray, N. Gadegaard, P. M. Tsimbouri, *et al*. Nanoscale surfaces for the long-term maintenance of mesenchymal stem cell phenotype and multipotency. *Nat Mater* 2011;**10**(8):637–644.

39 M. J. Dalby, N. Gadegaard, R. Tare, *et al*. The control of human mesenchymal cell differentiation using nanoscale symmetry and disorder. *Nat Mater* 2007;**6**(12):997–1003.

40 K. A. Kilian, B. Bugarija, B. T. Lahn, M. Mrksich. Geometric cues for directing the differentiation of mesenchymal stem cells. *Proc Natl Acad Sci U S Am* 2010;**107**(11):4872–4877.

41 C. S. Chen, M. Mrksich, S. Huang, G. M. Whitesides, D. Ingber. Micropatterned surfaces for control of cell shape, position, and function. *Biotechnol Prog* 1998;**14**(3):356–363.

42 R. McBeath, D. M. Pirone, C. M. Nelson, K. Bhadriraju, C. S. Chen. Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004;**6**(4):483–495.

43 F. Pampaloni, E. G. Reynaud, E. H. Stelzer. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* 2007;**8**(10):839–845.

44 A. M. Kloxin, A. M. Kasko, C. N. Salinas, K. S. Anseth. Photodegradable hydrogels for dynamic tuning of physical and chemical properties. *Science* 2009;**324**(5923):59–63.

45 Y. Tang, R. G. Rowe, E. L. Botvinick, *et al*. MT1-MMP-dependent control of skeletal stem cell commitment via a β1-integrin/YAP/TAZ signaling axis. *Dev Cell* 2013;**25**(4):402–416.

46 P. Meunier, J. Aaron, C. Edouard, G. Vignon. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. *Clin Orthop Relat Res* 1971;**80**:147–154.

CHAPTER 16

# Effects of hypoxic culture on bone marrow multipotent mesenchymal stromal cells: from bench to bedside

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## 16.1 Introduction

Human multipotent mesenchymal stromal cells (MSCs) isolated from human tissues such as bone marrow have become a promising tool for clinical applications. However, successful application of MSCs for therapeutic uses requires the development of techniques and protocols for the preparation of cell products. New techniques for expanding MSCs need to overcome cell senescence, loss of pluripotent gene expression, decreased self-renewal and differentiation potential, low engraftment after transplantation *in vivo*, and rejection by allogeneic recipients. Moreover, expanded MSCs should have a normal karyotype and genome integrity to prevent tumorigenic transformation. MSCs cultured under hypoxic conditions, the normal physiological state of the bone marrow, represent a new source of expanded MSCs for clinical applications. This chapter will address the effects of hypoxia on MSCs especially for the purpose of translation from bench to bedside and will emphasize that hypoxia is a key aspect of the biological profiles of MSCs.

## 16.2 Multipotent mesenchymal stromal cells

Multipotent MSCs are the best-studied subpopulations of stem/progenitor cells in the bone marrow, which are capable of differentiating into several nonhematopoietic cells after isolation from bone marrow by adherence to tissue culture surface [1–4].

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They are easily isolated, can be expanded in culture without the loss of differentiation potential and immune modulating properties, and have limited, if any, tendency to form tumors. Thus, MSCs have emerged as a promising tool for clinical applications such as tissue engineering and cell-based therapy. This first drug of stem/progenitor cells in the world was approved in Canada in May 2012 for the treatment of children with severe treatment-refractory acute graft-versus-host disease occurring after allogeneic hematopoietic stem transplantation.

## 16.3 Criteria for defining human multipotent stromal cells

Because the therapeutic potential of MSCs has generated significant increase in a wide variety of biomedical disciplines and because of difficulty in comparing the results of *ex vivo* expanded MSCs from one laboratory to another, the International Society for Cellular Therapy proposed minimum criteria to define human MSCs. The expanded MSCs for clinical applications must meet all the criteria [5]. First, the MSCs should be plastic adherent when maintained in standard culture conditions. Second, the MSCs must express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD19 or CD79α, and HLA-DR surface molecules. Third, the MSCs must possess the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*.

## 16.4 Problems encountered in the clinical application of multipotent mesenchymal stromal cells

There are several problems associated with the clinical application of MSCs (Table 16.1). For clinical applications these cells must be expanded in large scale using a gold-standard protocol without undergoing senescence or loss of proliferative capacity. Moreover, expanded cells must meet the criteria for MSCs, maintain mesodermal lineage differentiation potential, and graft into, or regenerate, tissues after their autologous or allogeneic transplantation. Since it has been proposed that MSC-derived microvesicles may protect against injuries or inflammation in injured tissues such as the kidney of the mouse remnant kidney model [6] or the cornea following chemical or mechanical injury [7], the actual functions of MSCs or their secreted factors – such as tumor necrosis factor-inducible protein 6 [8] in mediating therapeutic effects – should be clarified before clinical application. More importantly, processing procedures, instruments, or installation used for cell manipulation or expansion should be regulated and approved to provide products that pass the tests regarding the identity, strength, purity, and product safety of cell product (see United States Pharmacopeia 32 ⟨1046⟩ and Chapters 43 and 44).

**Table 16.1** Problems encountered in the clinical application of human multipotent stromal cells.

| |
|---|
| Expansion or scale-up |
| • no standard protocol for cell expansion |
| Phenotypes and functions change after expansion |
| • senescence and loss of proliferation<br>• lack of pluripotency gene expression<br>• loss of (trans-)differentiation and repairing ability<br>• decrease in "paracrine effects" |
| *In vivo* transplantation |
| • low engraftment and differentiation rate after transplantation<br>• rejection after allogeneic transplantation (HLA-mismatch) |
| Safety and regulation issues |
| • identify test<br>• potency test<br>• purity test<br>• safety test |

## 16.5 The hypoxic niche of multipotent mesenchymal stromal cells

The bone marrow niche facilitates the maintenance of hematopoietic stem cells (HSCs) as undifferentiated stem cells while supporting lineage commitment of the cells of the hematopoietic and immune systems [9]. MSCs residing in the bone marrow are associated with, and maintain, HSC functions and produce mesenchymal cells such as osteoblasts and adipocytes for maintaining the structure of bone. Bone marrow is hypoxic, with oxygen tension about 1 to 7% [10,11]. Because the general culture condition of 21% $O_2$ in the air of the incubator is hyperoxic compared with that of the normal bone marrow niche of MSCs, one strategy to solve the problems encountered in the clinical application of MSCs is expansion of these cells under hypoxic conditions. Therefore, the roles of the bone marrow niche in maintaining MSC properties and functions should be well investigated (see Chapter 32). For investigation of the effects of the marrow niche on MSC properties and functions we and other groups cultured MSCs under hypoxic conditions and compared the results with those obtained under normoxic conditions. Consistently, these studies have shown that MSCs cultured under hypoxia or absolute hypoxia meet the criteria for defining MSCs [5] without undergoing apoptosis or changes in immunophenotype [12,13]. Most of these studies also found that culture under hypoxic conditions had beneficial effects on the properties and functions of MSCs. This chapter aims to discuss the effects of hypoxic culture on MSC properties and functions, especially for their translation from bench to bedside.

## 16.6 Involvement of hypoxia-inducible factor-1α in hypoxia-mediated effects

Hypoxia is known to regulate various cellular processes, including signal transduction. Under hypoxic conditions, regulatory α subunits of hypoxia-inducible factors (HIFs) 1α and 2α are constitutively stabilized by escaping from oxygen-dependent and von Hippel–Lindau tumor suppressor-mediated ubiquitylation and proteasomal degradation [14–17], leading to increased levels of HIF-1 and HIF-2 and a wide range of hypoxia-inducible mRNAs, including those involved in energy metabolism, angiogenesis, and apoptosis, such as glucose transporter and vascular endothelial growth factor (VEGF). Most of the effects of HIF-1α have been investigated for cancer cells. Hypoxia or the hypoxic state, mimicked by the use of cobalt chloride ($CoCl_2$) and the iron chelator desferrioxamine, induced the accumulation of wild-type p53 by HIF-1α-dependent association with, and stabilization of, p53 protein [18]. (Tumor protein 53, or p53, is crucial in multicellular organisms, where it prevents cancer formation and thus functions as a tumor suppressor. p53 has been described as "the guardian of the genome" because of its role in conserving stability by preventing genome mutation. The International Cancer Genome Consortium established that the *TP53* gene is the most frequently mutated gene (>50%) in human cancers, indicating that the *TP53* gene plays a crucial role in preventing cancer formation. The *TP53* gene encodes proteins that bind to DNA and regulate gene expression to prevent mutations of the genome.)

HIF-1α blocks neuronal and myogenic differentiation via recruitment to Notch-responsive promoters after Notch activation under hypoxic conditions in neural stem cells and myogenic cells respectively [19]. Recently, HIF-1α was shown to regulate the proliferation of MSCs through enhanced TWIST expression, which downregulates the E2A–p21 pathway, and thus inhibits replicative senescence and stimulates proliferation [20]. It should be noted that HIF-1α upregulates the surface expression of chemokine (C–X–C motif) receptor 4 (CXCR4) and chemokine (C–X3–C motif) receptor 1 (CX3CR1) in MSCs, thereby enhancing their migration and engraftment after transplantation [21]. HIF-1α also plays an important role in the mobilization into the peripheral blood of hypoxia-induced MSCs, possibly acting via its downstream genes VEGF and stromal-cell-derived factor-1α (SDF-1α, also known as CXCL12) [22]. Recent studies have also suggested that HIF-1α and hypoxia-mimicking agents trigger the initiation of the angiogenic–osteogenic cascade and promote bone repair and regeneration [23]. Furthermore, networks of transcription factors related to HIF-1α and miR-124a in part control extensive changes of their global gene expression profile during the conversion of MSCs into neural stem cell-like cells [24]. Although HIF-2α is known to upregulate the expression of pluripotent genes such as *OCT4* [25], thereby maintaining embryo development, few studies have mentioned the involvement of HIF-2α in regulating the properties of MSCs. We have previously compared the protein levels of HIF-2α between MSCs cultured under normoxic and hypoxic conditions and found no difference in the level of HIF-2α between these two conditions [20].

## 16.7 Effects of hypoxic culture on glucose metabolism and oxidative stress of multipotent mesenchymal stromal cells

It has been shown that exposure to hypoxia or hypoxia-mimetic agents, such as $CoCl_2$, induces expression of HIF-1α and glucose-6-phosphate transporter (G6PT) in MSCs. Moreover, knockdown of HIF-1α inhibited the expression of G6PT in hypoxic MSCs, whereas constitutive expression of HIF-1α with a mutant oxygen-dependent degradation domain resulted in increased G6PT expression in normoxic MSCs, suggesting the involvement of the HIF pathway in G6PT expression [26]. Additionally, a potent G6PT inhibitor, AD4-015, specifically causes cell death in MSCs constitutively expressing the HIF-1α mutant. Collectively, these data suggest that G6PT may account for metabolic flexibility allowing MSCs to survive under conditions characterized by hypoxia [26]. MSCs cultured under hypoxic conditions also have a higher specific consumption of nutrients, especially at the beginning of culture, but exhibit a lower specific production of inhibitory metabolites. However, the hypoxic culture of MSCs has been shown to increase cell division and expansion for the progeny of colony-forming units–fibroblasts, while maintaining the characteristics of MSC-like immunophenotype and differentiation [27]. These data suggest that MSCs expanded under hypoxia favor increased cellular metabolism efficiency, leading to maximization of the yield of cells for application in clinical settings [27]. MSCs were also resistant to exposure to absolute hypoxia (0.5% $O_2$) and inhibition of mitochondrial respiration with 2,4-dinitrophenol [28], indicating that, in the absence of oxygen, MSCs may survive using anaerobic adenosine-5′-triphosphate production. Other studies have demonstrated that MSCs are able to survive hypoxia owing to their ability to rely on glycolysis instead of mitochondrial respiration [28]. Hypoxic preconditioning of MSCs also induces glycogen synthesis to provide additional sources of glycogen for glycolysis, thereby improving MSC survival and therapeutic effects in ischemic tissues [29]. Taken together, these studies suggest that MSCs are characterized by metabolic flexibility, allowing them to survive in conditions of hypoxia and ischemic stress, and enabling them to function in a restorative or regenerative capacity in the treatment of ischemic diseases.

## 16.8 Effects of hypoxic culture on the apoptosis of multipotent mesenchymal stromal cells

Significant apoptosis in MSCs has been demonstrated in conditions associated with the depletion of serum, especially during an extended period [30]. Most studies, however, have demonstrated that MSCs cultured under hypoxia or absolute hypoxia do not undergo apoptosis or changes in immunophenotype [12,13]. Moreover, the combination of exposure to hypoxia partly inhibited apoptosis induced by serum depletion [30,31]. The mechanisms that MSCs use to survive under hypoxic or ischemic conditions include the use of the glycolytic pathway [28] and the production of glucose-6-phosphatase for using glucose [26]. These results suggest that MSCs are characterized by metabolic flexibility, allowing them to survive from hypoxic and ischemic stress and retain their stem/progenitor cell phenotype. These results indicate the potential usefulness of MSCs in treating ischemic diseases.

The survival rate of MSCs at 4 days of transplantation into an ischemic heart was 0.44% [32]. Hypoxic preconditioning enhances the ability of MSCs to survive after transplantation into the ischemic heart [33–35] and limb tissues [29]. Similarly, hypoxic preconditioning of neural stem cells and MSCs also increased their survival and decreased the activation of caspase-3 after their transplantation into ischemic brain [36]. One proposed mechanism is that hypoxia-induced glycogen-synthesis-associated metabolic adaptation promotes the survival of MSCs in long-term ischemia [29]. These data suggest that short-term exposure of MSCs to hypoxia prior to transplantation improves survival rates following transplantation. Although hypoxic preconditioning increases survival, MSCs still cannot stay long in ischemic tissues, conditions associated with hypoxia and lack of nutrition [30]. To overcome these obstacles, several measures have been shown to help MSCs survive under these conditions, including transfection of MSCs with plasmids carrying the integrin-linked kinase [37], heme oxygenase-1 (HO-1) [38], angiogenin [39], angiopoietin-1, Akt [40], and the 70 kDa heat-shock protein [41], or pretreatment of MSCs with cyclosporine [42] or lysophosphatidic acid [43]. Most of these measures increased the survival of MSCs through downregulating pathways associated with apoptosis. In addition, HO-1 significantly affected the potential of cell differentiation through suppression of the microRNAs miR-1, miR-133a, miR-133b, and miR-206 [44].

## 16.9 Effects of hypoxic culture on expansion and life span of multipotent mesenchymal stromal cells

The effects of hypoxia on the proliferation and expansion efficiency of MSCs depend on the sources from which the MSCs are derived, the oxygen concentration, the seeding density, and the duration of time used for culture (Table 16.2).

Some studies found that hypoxia significantly inhibited proliferation [21,45,46,49,51], while others found that hypoxia increased proliferation or increased the culture duration for approaching senescence [12,45,48,50,53–56]. We have shown that hypoxia inhibited proliferation at the earliest passages of culture either at low density (10–200 cells/cm$^2$) or at high density (1000–4000 cells/cm$^2$) [21], while hypoxia significantly increased the efficiency of expansion in long-term culture (up to 60 days) of MSCs at low density (Table 16.3) [20].

Moreover, hypoxia also inhibited senescence, increased proliferation, and enhanced differentiation potential *in vivo* and *in vitro* [20]. Together, these results show that the effects of

**Table 16.2** Comparisons of effects of hypoxia on the proliferation of multipotent stromal cells.

| MSC source | Effects on proliferation | $O_2$ (%) or hypoxia micmicking; seeding density; period tested | Reference |
|---|---|---|---|
| BM, murine | Promotion | 8%; 10 000; 7–8 days | [45] |
| BM, murine | Inhibition | $CoCl_2$ or DFX; 10 000; 7–8 days | [45] |
| BM, human | Inhibition | 1%; 50–10 000, 10 days | [21] |
| BM, human | Inhibition | 1–5%; 6250; 7 days | [46] |
| BM, human | Promotion | 1–7%; 50; 2–7 passages | [20] |
| AT, murine | Promotion | 2%; 10 000; 13 days | [47] |
| AT, human | Promotion | 2%; 3000; 14 days | [48] |
| BM and AT, canine | Inhibition | 1%, 5%; 5000; 7–14 days | [49] |
| WJ, human | Promotion | 2%; 5000; 10 passages | [50] |
| PDB, human | Inhibition | 1%; 10 000; 2–4 days | [51] |

*Source*: [52].
BM, bone marrow; AT, adipose tissue; WJ, Wharton's jelly; PDB, placental decidua basalis; DFX, desferoxamine.

**Table 16.3** Efficiency of MSC expansion under different seeding density and culture conditions.

| Hypoxia or normoxia | Seeding density (cells/cm$^2$) | Subculture periods (days) | Passages for 60 days | Cell fold increase for each passage | Expected cell fold increase for 60 days | Ratio to Nor/4000 |
|---|---|---|---|---|---|---|
| Nor | 4000 | 5–7 | 10 | 5 for each passage | 9 765 625 | 1 |
| Nor | 1000 | ~10 | 6 | 18, 18, 16, 15, 14, 11 | 11 975 040 | 1.2 |
| Nor | 100 | 11–12 | 5 | 190, 121, 68, 45, 40 | 2 813 976 000 | 288.2 |
| Nor | 50 | ~12 | 5 | 252, 170, 80, 70, 48 | 11 515 392 000 | 1179.20 |
| Hyp (1%) | 50 | ~12 | 5 | ~250 for each passage | 976 562 500 000 | 100 000 |

*Source*: [52].

hypoxia on the short-term proliferation of MSCs varied and depended on the oxygen tension, seeding densities, and sources from which the MSCs were isolated. The beneficial effects of hypoxia on long-term expansion of MSCs are consistent even with different sources of MSCs in a variety of concentrations of oxygen [12,20,50].

## 16.10 Effects of hypoxic culture on maintaining self-renewal and differentiation potential of multipotent mesenchymal stromal cells

Proliferation and differentiation potential are important hallmarks of viable multipotent MSCs. Low-density culture under normoxic conditions provides a method for the rapid expansion of MSCs [57] at the expense of a loss in proliferation ability and differentiation potential [20,58]. Furthermore, long-term expansion of MSCs under normoxia adversely affects cell function when MSCs were applied for protecting the myocardium [59]. In contrast, the combination of low-density culture with hypoxic culture maintains proliferation and differentiation potential, increases the expression of pluripotency genes, such as *OCT4*, *NANOG*, and *SOX2* [60], and inhibits senescence [20]. Similarly, MSCs with hypoxic preconditioning have been shown to exhibit increased differentiation into bone [61], fat, and cartilage lineages [62] both *in vitro* and *in vivo* [20]. Hypoxic MSCs have also been observed to increase telomere length and telomerase activity, and have normal karyotyping and chromosomal integration [20]. Moreover, MSCs with long-term hypoxic preconditioning do not form tumors when transplanted into immunodeficient mice. These results suggest that the hypoxic culture provides a method for expanding MSCs efficiently without losing stemness [60,63] or increasing tumorigenicity. Notably, the effects of hypoxic culture on the maintenance of self-renewal and differentiation potential and inhibition of senescence of expanded stem cells have also been observed in MSCs derived from amniotic fluid [64], adipose tissue [48], umbilical cord [65,66], and endothelial progenitor cells [67]. Of note, however, is the fact that multipotent MSCs have not shown any proclivity to form tumors, regardless of culture conditions and regardless of their tissue source. In this respect they behave quite differently to embryonic stem cells and to induced pluripotent stem cells, in which teratoma formation is one criterion that indicates multipotency or totipotency.

## 16.11 Differentiation of multipotent mesenchymal stromal cells under hypoxic conditions

It is well documented that hypoxic culture inhibits osteogenic and adipogenic differentiation of bone-marrow-derived MSCs [12,46,68]. The same effects have also been shown for adipose-tissue-derived MSCs [68]. Compared with normoxic cultures, hypoxic cultures with induction of MSCs to osteogenic differentiation show decreased expression of osteogenic genes, such as *RUNX2* and osteocalcin, and reduced alkaline phosphatase activity and mineralization capacity [12,13,46,68,69]. The mechanism is mediated through downregulation of the transcription factor RUNX2 by the activation of the HIF–TWIST pathway (Figure 16.1) [70].

In particular, hypoxia or activation of HIF-1α also improved *in vivo* osteoclastogenesis [71,72]. Similarly, MSCs induced in adipogenic medium under hypoxic conditions show decreased expression of adipogenic genes, such as *PPAR* and *LPL*, and a reduced ability to accumulate lipid droplets [12,46]. However, MSCs that were induced for chondrogenesis under hypoxic conditions show increased expression of chondrogenic genes, such as *SOX5*, *-6*, and *-9*, aggrecan, and type II collagen [73–77], decreased expression of genes associated with endochondral bone formation, such as *COL-1a1* and *COL-10a1*, as well as suppression of interleukin (IL)-1β-induced loss of extracellular matrix proteins [74]. One of the mechanisms by which hypoxia mediates enhanced chondrogenesis and inhibits endochondral ossification of MSCs is through the activation of PI3K/AKT/FOXO-dependent anti-apoptotic effect [78]. Similarly, hypoxic culture has also been applied for inducing MSCs to differentiate into nucleus pulposus-like cells both *in vitro* [79] and *in vivo* [65].

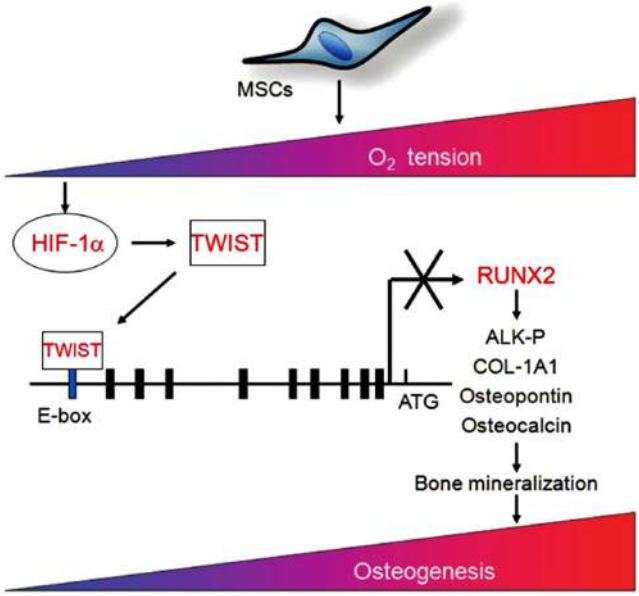



**Figure 16.1** A schematic representation showing the underlying mechanism that hypoxia mediates to downregulate RUNX2 expression and inhibit osteogenesis in bone marrow MSCs [70]. *Source:* adapted from *Am J Blood Res* 2012, **2**, pp. 148–159 [52].

Furthermore, hypoxic culture and agents that mimic hypoxia have been shown to increase the ectodermal differentiation of neuron-like cells [80] and the regenerative potential of the skin [81]. A prolyl hydroxylase inhibitor, FG-0041, in combination with an inhibitor of ROCK, Y-27632, showed initiation of differentiation of MSCs into neuron-like cells [82].

## 16.12 Effects of hypoxic culture on secretion of paracrine factors by multipotent mesenchymal stromal cells

It has been proposed that the properties of endogenous MSCs in the bone marrow of mice with recent myocardial infarction (MI) changed along with the exposure to hypoxia and inflammation. Wang *et al.* demonstrated that the therapeutic efficacy of bone marrow cells was impaired by a recent MI. MI led to inflammation of the myocardium and an increased inflammatory state in the bone marrow, thereby changing the composition of bone marrow cells and reducing its effectiveness. Injection of a general anti-inflammatory drug or a specific IL-1 inhibitor to mice after MI prevented this deterioration [83]. In contrast, *in vitro* hypoxic preconditioning enhanced the ability of MSCs to repair infarcted myocardium or diabetic cardiomyopathy. This is attributable to reduced apoptosis and cell death of the implanted cells, increased angiogenesis/vascularity, and enhanced paracrine effects [33–35]. It has been demonstrated that conditioned medium (CM) of MSCs from mice overexpressing the antiapoptotic proto-oncogene *AKT* markedly inhibited apoptosis induced by hypoxia and triggered vigorous spontaneous contraction of adult rat cardiomyocytes *in vitro* [84]. Similarly, MSC CM has also been observed to reduce apoptosis or enhance tube formation in human endothelial cells [31]. Moreover, the effects were more evident in the CM of MSCs cultured in hypoxic conditions compared with those of MSCs cultured in normoxic conditions. This is due in part to their higher content of antiapoptotic and angiogenic factors, including IL-6, VEGF [33,64,85], transforming growth factor-β1 [64], fibroblast growth factor 2, insulin-like growth factor 1, brain-derived growth factor, glial-cell-derived neurotrophic factor, and hepatocyte growth [86]. Analysis of gene expression profiles using microarrays also showed that MSCs cultured in hypoxic conditions increased expression of various growth factors involved in cell proliferation, apoptosis, and angiogenesis [87]. In addition, the CM of MSCs grown in hypoxic conditions activated the PI3K–AKT pathway in endothelial cells and thus inhibited hypoxia-induced apoptosis and increased angiogenesis of endothelial cells [32]. The *in vivo* cardioprotection effect of the CM of hypoxic MSCs was tested in a model of MI induced in Wistar male rats by permanent occlusion of the left coronary artery. Intramyocardial injection of 25× concentrated CM 3 h after coronary occlusion was able to promote a significant reduction (35%) in the left ventricular end-diastolic pressure and improve cardiac contractility (15%) and relaxation (12%) compared with unconditioned medium 19–21 days after the medium was injected [88]. These results suggest that soluble factors released by MSCs *in vitro* are able to promote cardioprotection and improve cardiac function *in vivo*. Additionally, CM of human hypoxic MSCs also enhanced wound healing in the skin [89] and small bowel lesions induced by radiation in mice [90] and promoted fracture healing in diabetic rats [91]. Together, these results suggest that administration of hypoxic MSCs or their secreted factors provides a therapeutic method for enhancing angiogenesis, epithelialization, wound healing, and fracture repair (Figure 16.2).

Furthermore, hypoxic MSCs, but not normoxic MSCs, restored conduction velocities, suppressed focal activity, and prevented sudden arrhythmic deaths when delivered to the border zone of infarcted myocardium in rats [92]. Hypoxic MSCs have also been demonstrated to ameliorate atherosclerotic lesions both *in vitro* and *in vivo* by secreting IL-8/MIP2 [93]. (MIP2, macrophage inflammatory protein, is the murine homolog of IL-8.)

In addition to the increase in the secretion of paracrine factors, hypoxic MSCs have also been shown to increase levels of total protein and different patterns of fibronectin expression throughout the culture period, suggesting that oxygen levels can significantly affect tissue development patterns [94]. The mechanism that hypoxic MSCs use to increase the release of angiogenic factors such as VEGF is partly through the HIF-1α pathway [95]. Moreover, the advantages of using CM instead of cells are twofold: (1) CM-based therapy avoids some of the problems

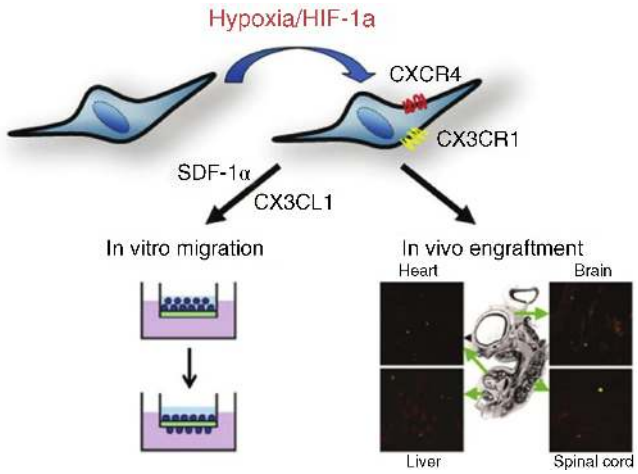



**Figure 16.2** A schematic representation summarizing the effects of hypoxic exposure on enhancing the expression of chemokine receptors and increasing *in vitro* migration and *in vivo* engraftment in bone marrow MSCs [21]. *Source:* adapted from *Am J Blood Res* 2012, **2**, pp. 148–159 [52].

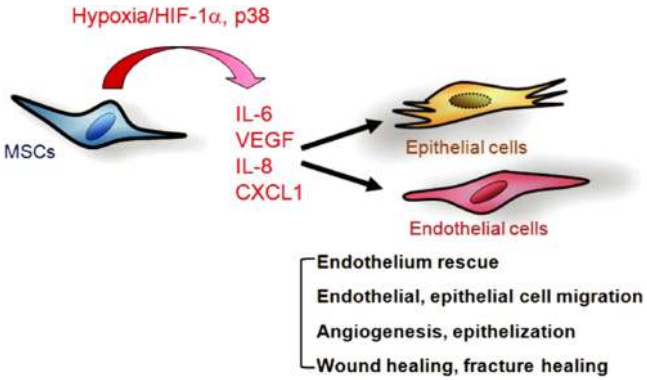



**Figure 16.3** A schematic representation summarizing the effects of hypoxic culture on enhancing secretion of paracrine factors and promoting therapeutic effects in bone marrow MSC [31,89,91]. *Source:* adapted from *Am J Blood Res* 2012, **2**, pp. 148–159 [52].

and limitations associated with viable and nonreplicating cells and does not compromise all of the advantages associated with the use of cells; (2) CM-based therapy is an ideal therapeutic approach because the complex load of proteins and genetic material has the diversity and biochemical potential to participate in multiple biochemical and cellular processes, an important attribute in the treatment of complex diseases [96].

## 16.13 Effects of hypoxic culture on engraftment of multipotent mesenchymal stromal cells

The capacity of MSCs for homing to, and engraftment in, target tissues after transplantation is the key to success in their clinical application. The degree of homing and engraftment of MSCs in adult recipients is very low [97–99]. Interestingly, it has been shown that short-term (more than 1 day) exposure of MSCs to hypoxia, such as 1% $O_2$ or hypoxic preconditioning of MSCs, increases *in vitro* migration, *in vivo* engraftment, and therapeutic effects in stroke or other ischemic tissues by an increase in CX3CR1–Fractalkine and CXCR4–SDF-1α interactions (Figure 16.3) [21,100], CXCR7 expression [101] or the upregulation of matrix metalloproteinase (MMP) 2, MMP-9 [100], $K^+$ channel, $K_v2.1$ [102] or Wnt4 [103], and FAK activation [102].

MSCs cultured under hypoxic conditions also increased the expression of VEGF and VEGFR1 or placental growth-factor-dependent migration [104]. Moreover, hypoxia-dependent MSC migration relies on a biphasic scenario involving an early phase, requiring generation of reactive oxygen species, and a delayed phase sustained by HIF-dependent expression of VEGF [105]. Hypoxia preconditioning when combined with glucose depletion also increased the survival of Sca-$1^+$ cells via PI3K/AKT-dependent connexin-43 upregulation and caspase-3 downregulation, and thereby increased the engraftment rate [106]. In addition to increased migration and survival, MSCs incubated in hypoxic conditions or in a hypoxic microenvironment such as cell aggregates have also been shown to enhance revascularization after transplantation for hind limb ischemia [107,108]. These results suggest that short-term hypoxic preconditioning of MSCs may provide a method of improving their survival, migration, angiogenesis, and engraftment *in vivo* into a variety of tissues.

## 16.14 Effects of hypoxic culture on allogeneic transplantation of multipotent mesenchymal stromal cells

The use of MSCs from healthy donors unrelated to, and major histocompatibility complex (MHC)-unmatched with, the MSC recipient as an "off-the-shelf" cellular product has been shown to be safe in multiple clinical trials (http://www.clinicaltrails.gov/). Additionally, it has been reported that such allogeneic MSCs improved the ejection fraction in patients after acute myocardial infarction [109]. The immunosuppressive properties of human, murine, and baboon MSCs have been demonstrated both *in vitro* [110–113] and *in vivo* [113], and human MSCs have been shown to ameliorate severe acute graft-versus-host disease in recipients of HSC transplants [114].

Two studies using murine MSCs and one using human MSCs, however, showed that allogeneic MSCs were rejected in MHC-mismatched recipients [115–117]; and see Chapter 29. Therefore, the use of MHC-unmatched MSCs for allogeneic transplantation to immune-competent recipients remains controversial, although the widespread use of autologous MSC production is not commercially feasible at the present time. The identification of methods to increase the immune privilege

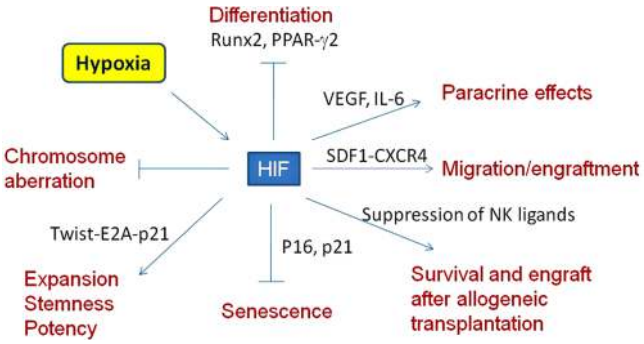



**Figure 16.4** A schematic representation summarizing the effects of hypoxic culture on proliferation, expansion, differentiation, engraftment, stem cell properties, and resistance for immune rejection by allogeneic recipients in bone marrow MSCs [20,21,31,60,70,89,91,117,118,119]. The mechanism that hypoxia mediates to suppress NK ligands is still unknown. *Source:* adapted from *Am J Blood Res* 2012, **2**, pp. 148–159 [52].

of MSCs so that they do indeed become a "true universal donor cell" is important. One study showed that allogeneic hypoxic MSCs increased the therapeutic effects in a bone-defect repair model compared with allogeneic normoxic MSCs [118]. The superiority of hypoxic MSCs to normoxic MSCs for allogeneic transplantation was also demonstrated when applied to animal models of tendon injury [119] and ischemic limb injury [120], as well as for xenograft transplantation in a swine model of chronic MI [121]. Hypoxic MSCs escape immune rejection by allogeneic recipients through suppression of NK ligand expression during hypoxic preconditioning [119]. These results suggest that hypoxic MSCs are intrinsically immunoprivileged and may be a more clinically effective "universal donor cell" than MSCs grown in normoxic conditions.

## 16.15 Conclusions

Currently available studies suggest that hypoxic cultures have multiple effects on MSCs, affecting short-term proliferation, long-term expansion efficiency, differentiation potential, stemness or maintenance of stem cell properties, the expression of chemokine receptors, migration, and engraftment ability. Moreover, MSCs are able to survive and engraft in allogeneic recipients. Notably, most of the effects are mediated through the HIF-1α-related pathways (Figure 16.4).

A manufacturing platform based on hypoxic cultures of MSCs help develop more effective strategies for their clinical application.

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## References

1 M. Owen, A. J. Friedenstein. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* 1988;**136**:42–60.
2 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.
3 D. J. Prockop. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;**276**:71–74.
4 D. J. Prockop, C. A. Gregory, J. L. Spees. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. *Proc Natl Acad Sci U S A* 2003;**100**(Suppl 1):11917–11923.
5 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–347.
6 J. He, Y. Wang, S. Sun, *et al.* Bone marrow stem cells-derived microvesicles protect against renal injury in the mouse remnant kidney model. *Nephrology (Carlton)* 2012;**17**:493–500.
7 J. Y. Oh, G. W. Roddy, H. Choi, *et al.* Anti-inflammatory protein TSG-6 reduces inflammatory damage to the cornea following chemical and mechanical injury. *Proc Natl Acad Sci U S A* 2010;**107**:16875–16880.
8 R. H. Lee, J. M. Yu, A. M. Foskett, *et al.* TSG-6 as a biomarker to predict efficacy of human mesenchymal stem/progenitor cells (hMSCs) in modulating sterile inflammation in vivo. *Proc Natl Acad Sci U S A* 2014;**111**:16766–16771.
9 Y. Shiozawa, A. M. Havens, K. J. Pienta, R. S. Taichman. The bone marrow niche: habitat to hematopoietic and mesenchymal stem cells, and unwitting host to molecular parasites. *Leukemia* 2008;**22**:941–950.
10 D. C. Chow, L. A. Wenning, W. M. Miller, E. T. Papoutsakis. Modeling $pO_2$ distributions in the bone marrow hematopoietic compartment. I. Krogh's model. *Biophys J* 2001;**81**:675–684.
11 D. C. Chow, L. A. Wenning, W. M. Miller, E. T. Papoutsakis. Modeling $pO_2$ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. *Biophys J* 2001;**81**:685–696.
12 C. Fehrer, R. Brunauer, G. Laschober, *et al.* Reduced oxygen tension attenuates differentiation capacity of human mesenchymal stem cells and prolongs their lifespan. *Aging Cell* 2007;**6**:745–757.
13 A. Salim, R. P. Nacamuli, E. F. Morgan, *et al.* Transient changes in oxygen tension inhibit osteogenic differentiation and *Runx2* expression in osteoblasts. *J Biol Chem* 2004;**279**:40007–40016.
14 K. Tanimoto, Y. Makino, T. Pereira, L. Poellinger. Mechanism of regulation of the hypoxia-inducible factor-1α by the von Hippel–Lindau tumor suppressor protein. *EMBO J* 2000;**19**:4298–4309.
15 M. Ohh, C. W. Park, M. Ivan, *et al.* Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel–Lindau protein. *Nat Cell Biol* 2000;**2**:423–427.
16 M. E. Cockman, N. Masson, D. R. Mole, *et al.* Hypoxia inducible factor-α binding and ubiquitylation by the von Hippel–Lindau tumor suppressor protein. *J Biol Chem* 2000;**275**:25733–25741.
17 P. H. Maxwell, M. S. Wiesener, G. W. Chang, *et al.* The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. *Nature* 1999;**399**:271–275.
18 W. G. An, M. Kanekal, M. C. Simon, *et al.* Stabilization of wild-type p53 by hypoxia-inducible factor 1α. *Nature* 1998;**392**:405–408.

19 M. V. Gustafsson, X. Zheng, T. Pereira, *et al.* Hypoxia requires notch signaling to maintain the undifferentiated cell state. *Dev Cell* 2005;**9**:617–628.

20 C. C. Tsai, Y. J. Chen, T. L. Yew, *et al.* Hypoxia inhibits senescence and maintains mesenchymal stem cell properties through down-regulation of E2A–p21 by HIF–TWIST. *Blood* 2011;**117**:459–469.

21 S. C. Hung, R. R. Pochampally, S. C. Hsu, *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* 2007;**2**:e416.

22 L. Liu, Q. Yu, J. Lin, *et al.* Hypoxia-inducible factor-1α is essential for hypoxia-induced mesenchymal stem cell mobilization into the peripheral blood. *Stem Cells Dev* 2011;**20**:1961–1971.

23 A. A. Mamalis, D. L. Cochran. The therapeutic potential of oxygen tension manipulation via hypoxia inducible factors and mimicking agents in guided bone regeneration. *A review. Arch Oral Biol* 2011;**56**:1466–1475.

24 M. Maisel, H. J. Habisch, L. Royer, *et al.* Genome-wide expression profiling and functional network analysis upon neuroectodermal conversion of human mesenchymal stem cells suggest HIF-1 and miR-124a as important regulators. *Exp Cell Res* 2010;**316**: 2760–2778.

25 K. L. Covello, J. Kehler, H. Yu, *et al.* HIF-2α regulates *Oct-4*: effects of hypoxia on stem cell function, embryonic development, and tumor growth. *Genes Dev* 2006;**20**:557–570.

26 S. Lord-Dufour, I. B. Copland, L. C. Levros, Jr, *et al.* Evidence for transcriptional regulation of the glucose-6-phosphate transporter by HIF-1α: targeting G6PT with mumbaistatin analogs in hypoxic mesenchymal stromal cells. *Stem Cells* 2009;**27**: 489–497.

27 F. Dos Santos, P. Z. Andrade, J. S. Boura, *et al. Ex vivo* expansion of human mesenchymal stem cells: a more effective cell proliferation kinetics and metabolism under hypoxia. *J Cell Physiol* 2010;**223**: 27–35.

28 L. A. Mylotte, A. M. Duffy, M. Murphy, *et al.* Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. *Stem Cells* 2008;**26**:1325–1336.

29 H. Zhu, A. Sun, Y. Zou, J. Ge. Inducible metabolic adaptation promotes mesenchymal stem cell therapy for ischemia: a hypoxia-induced and glycogen-based energy prestorage strategy. *Arterioscler Thromb Vasc Biol* 2014;**34**:870–976.

30 E. Potier, E. Ferreira, A. Meunier, *et al.* Prolonged hypoxia concomitant with serum deprivation induces massive human mesenchymal stem cell death. *Tissue Eng* 2007;**13**:1325–1331.

31 S. C. Hung, R. R. Pochampally, S. C. Chen, *et al.* Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K–Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 2007;**25**:2363–2370.

32 C. Toma, M. F. Pittenger, K. S. Cahill, *et al.* Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002;**105**:93–98.

33 X. Hu, S. P. Yu, J. L. Fraser, *et al.* Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg* 2008;**135**:799–808.

34 J. H. Li, N. Zhang, J. A. Wang. Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. *J Endocrinol Invest* 2008;**31**:103–110.

35 J. A. Wang, A. He, X. Hu, *et al.* Anoxic preconditioning: a way to enhance the cardioprotection of mesenchymal stem cells. *Int J Cardiol* 2009;**133**:410–412.

36 M. H. Theus, L. Wei, L. Cui, *et al. In vitro* hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain. *Exp Neurol* 2008;**210**:656–670.

37 S. W. Song, W. Chang, B. W. Song, *et al.* Integrin-linked kinase is required in hypoxic mesenchymal stem cells for strengthening cell adhesion to ischemic myocardium. *Stem Cells* 2009;**27**: 1358–1365.

38 Y. L. Tang, Y. Tang, Y. C. Zhang, *et al.* Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *J Am Coll Cardiol* 2005;**46**:1339–1350.

39 X. H. Liu, C. G. Bai, Z. Y. Xu, *et al.* Therapeutic potential of angiogenin modified mesenchymal stem cells: angiogenin improves mesenchymal stem cells survival under hypoxia and enhances vasculogenesis in myocardial infarction. *Microvasc Res* 2008;**76**:23–30.

40 S. Jiang, H. Kh. Haider, N. M. Idris, *et al.* Supportive interaction between cell survival signaling and angiocompetent factors enhances donor cell survival and promotes angiomyogenesis for cardiac repair. *Circ Res* 2006;**99**:776–784.

41 W. Chang, B. W. Song, S. Lim, *et al.* Mesenchymal stem cells pretreated with delivered Hph-1–Hsp70 protein are protected from hypoxia-mediated cell death and rescue heart functions from myocardial injury. *Stem Cells* 2009;**27**:2283–2292.

42 T. L. Chen, J. A. Wang, H. Shi, *et al.* Cyclosporin A pre-incubation attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. *Scand J Clin Lab Invest* 2008;**68**:585–593.

43 J. Chen, A. R. Baydoun, R. Xu, *et al.* Lysophosphatidic acid protects mesenchymal stem cells against hypoxia and serum deprivation-induced apoptosis. *Stem Cells* 2008;**26**:135–145.

44 M. Kozakowska, M. Ciesla, A. Stefanska, *et al.* Heme oxygenase-1 inhibits myoblast differentiation by targeting myomirs. *Antioxid Redox Signal* 2012;**16**:113–127.

45 H. Ren, Y. Cao, Q. Zhao, *et al.* Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. *Biochem Biophys Res Commun* 2006;**347**:12–21.

46 C. Holzwarth, M. Vaegler, F. Gieseke, *et al.* Low physiologic oxygen tensions reduce proliferation and differentiation of human multipotent mesenchymal stromal cells. *BMC Cell Biol* 2010;**11**:11.

47 M. G. Valorani, A. Germani, W. R. Otto, *et al.* Hypoxia increases Sca-1/CD44 co-expression in murine mesenchymal stem cells and enhances their adipogenic differentiation potential. *Cell Tissue Res* 2010;**341**:111–120.

48 M. G. Valorani, E. Montelatici, A. Germani, *et al.* Pre-culturing human adipose tissue mesenchymal stem cells under hypoxia increases their adipogenic and osteogenic differentiation potentials. *Cell Prolif* 2012;**45**:225–238.

49 D. J. Chung, K. Hayashi, C. A. Toupadakis, *et al.* Osteogenic proliferation and differentiation of canine bone marrow and adipose tissue derived mesenchymal stromal cells and the influence of hypoxia. *Res Vet Sci* 2012;**92**:66–75.

50 U. Nekanti, S. Dastidar, P. Venugopal, *et al.* Increased proliferation and analysis of differential gene expression in human Wharton's

jelly-derived mesenchymal stromal cells under hypoxia. *Int J Biol Sci* 2010;**6**:499–512.
51 Y. C. Huang, X. H. Chen, J. Wang, *et al.* Effects of hypoxia on human placental decidua basalis-mesenchymal stem cells proliferation, apoptosis and VEGF expression. *Sheng Li Xue Bao* 2008;**60**:783–789 (in Chinese).
52 C.-C. Tsai, T.-L. Yew, D.-C. Yang, *et al.* Benefits of hypoxic culture on bone marrow multipotent stromal cells. *Am J Blood Res* 2012;**2**:148–159.
53 L. B. Buravkova, O. S. Grinakovskaia, E. P. Andreeva, *et al.* Characteristics of human lipoaspirate-isolated mesenchymal stromal cells cultivated under a lower oxygen tension. *Tsitologiia* 2009;**51**:5–11 (in Russian).
54 S. Carrancio, N. López-Holgado, F. M. Sánchez-Guijo, *et al.* Optimization of mesenchymal stem cell expansion procedures by cell separation and culture conditions modification. *Exp Hematol* 2008;**36**:1014–1021.
55 L. B. Buravkova, E. B. Anokhina. Effect of hypoxia on stromal precursors from rat bone marrow at the early stage of culturing. *Bull Exp Biol Med* 2007;**143**:411–413.
56 Y. C. Huang, Z. M. Yang, N. G. Jiang, *et al.* Characterization of MSCs from human placental decidua basalis in hypoxia and serum deprivation. *Cell Biol Int* 2010;**34**:237–243.
57 I. Sekiya, B. L. Larson, J. R. Smith, *et al.* Expansion of human adult stem cells from bone marrow stroma: conditions that maximize the yields of early progenitors and evaluate their quality. *Stem Cells* 2002;**20**:530–541.
58 Y. Jin, T. Kato, M. Furu, *et al.* Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. *Biochem Biophys Res Commun* 2010;**391**:1471–1476.
59 P. R. Crisostomo, M. Wang, G. M. Wairiuko, *et al.* High passage number of stem cells adversely affects stem cell activation and myocardial protection. *Shock* 2006;**26**:575–580.
60 C. C. Tsai, P. F. Su, Y. F. Huang, *et al. Oct4* and *Nanog* directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Molecular Cell* 2012.
61 D. P. Lennon, J. M. Edmison, A. I. Caplan. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol* 2001;**187**:345–355.
62 E. Martin-Rendon, S. J. Hale, D. Ryan, *et al.* Transcriptional profiling of human cord blood $CD133^+$ and cultured bone marrow mesenchymal stem cells in response to hypoxia. *Stem Cells* 2007;**25**:1003–1012.
63 W. L. Grayson, F. Zhao, B. Bunnell, T. Ma. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;**358**:948–953.
64 E. K. Jun, Q. Zhang, B. S. Yoon, *et al.* Hypoxic conditioned medium from human amniotic fluid-derived mesenchymal stem cells accelerates skin wound healing through TGF-beta/SMAD2 and PI3K/Akt pathways. *Int J Mol Sci* 2014;**15**:605–628.
65 A. Lavrentieva, I. Majore, C. Kasper, R. Hass. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. *Cell Commun Signal* 2010;**8**:18.
66 W. P. Tsang, Y. Shu, P. L. Kwok, *et al.* $CD146^+$ human umbilical cord perivascular cells maintain stemness under hypoxia and as a cell source for skeletal regeneration. *PLoS ONE* 2013;**8**:e76153.
67 S. H. Lee, J. H. Lee, S. Y. Yoo, *et al.* Hypoxia inhibits cellular senescence to restore the therapeutic potential of old human endothelial progenitor cells via the hypoxia-inducible factor-1α–TWIST-p21 axis. *Arterioscler Thromb Vasc Biol* 2013;**33**:2407–2414.
68 P. Malladi, Y. Xu, M. Chiou, *et al.* Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. *Am J Physiol Cell Physiol* 2006;**290**:C1139–C1146.
69 E. Potier, E. Ferreira, R. Andriamanalijaona, *et al.* Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* 2007;**40**:1078–1087.
70 D. C. Yang, M. H. Yang, C. C. Tsai, *et al.* Hypoxia inhibits osteogenesis in human mesenchymal stem cells through direct regulation of RUNX2 by TWIST. *PLoS ONE* 2011;**6**:e23965.
71 A. Bozec, L. Bakiri, A. Hoebertz, *et al.* Osteoclast size is controlled by Fra-2 through LIF/LIF-receptor signalling and hypoxia. *Nature* 2008;**454**:221–225.
72 Knowles HJ. Hypoxic regulation of osteoclast differentiation and bone resorption activity. The Dove Press, 2015, 73–82. doi: http://dx.doi.org/10.2147//HP.S95960.
73 B. D. Markway, G. K. Tan, G. Brooke, *et al.* Enhanced chondrogenic differentiation of human bone marrow-derived mesenchymal stem cells in low oxygen environment micropellet cultures. *Cell Transplant* 2010;**19**:29–42.
74 T. Felka, R. Schäfer, B. Schewe, *et al.* Hypoxia reduces the inhibitory effect of IL-1β on chondrogenic differentiation of FCS-free expanded MSC. *Osteoarthritis Cartilage* 2009;**17**:1368–1376.
75 M. Kanichai, D. Ferguson, P. J. Prendergast, V. A. Campbell. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1α. *J Cell Physiol* 2008;**216**:708–715.
76 W. S. Khan, A. B. Adesida, T. E. Hardingham. Hypoxic conditions increase hypoxia-inducible transcription factor 2α and enhance chondrogenesis in stem cells from the infrapatellar fat pad of osteoarthritis patients. *Arthritis Res Ther* 2007;**9**:R55.
77 Y. Kassir, G. Simchen. Monitoring meiosis and sporulation in *Saccharomyces cerevisiae*. *Methods Enzymol* 1991;**194**:94–110.
78 H. H. Lee, C. C. Chang, M. J. Shieh, *et al.* Hypoxia enhances chondrogenesis and prevents terminal differentiation through PI3K/Akt/FoxO dependent anti-apoptotic effect. *Sci Rep* 2013;**3**:2683.
79 Z. Fang, Q. Yang, W. Luo, *et al.* Differentiation of GFP-Bcl-2-engineered mesenchymal stem cells towards a nucleus pulposus-like phenotype under hypoxia *in vitro*. *Biochem Biophys Res Commun* 2013;**432**:444–450.
80 E. Pacary, H. Legros, S. Valable, *et al.* Synergistic effects of $CoCl_2$ and ROCK inhibition on mesenchymal stem cell differentiation into neuron-like cells. *J Cell Sci* 2006;**119**:2667–2678.
81 H. M. Chung, C. H. Won, J. H. Sung. Responses of adipose-derived stem cells during hypoxia: enhanced skin-regenerative potential. *Expert Opin Biol Ther* 2009;**9**:1499–1508.
82 E. Pacary, E. Petit, M. Bernaudin. Concomitant inhibition of prolyl hydroxylases and ROCK initiates differentiation of mesenchymal stem cells and PC12 towards the neuronal lineage. *Biochem Biophys Res Commun* 2008;**377**:400–406.
83 X. Wang, J. Takagawa, V. C. Lam, *et al.* Donor myocardial infarction impairs the therapeutic potential of bone marrow cells by an interleukin-1-mediated inflammatory response. *Sci Transl Med* 2011;**3**:100ra90.

84 M. Gnecchi, H. He, N. Noiseux, *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* 2006;**20**:661–669.

85 L. Wei, J. L. Fraser, Z. Y. Lu, *et al.* Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiol Dis* 2012;**46**:635–645.

86 P. R. Crisostomo, Y. Wang, T. A. Markel, *et al.* Human mesenchymal stem cells stimulated by TNF-α, LPS, or hypoxia produce growth factors by an NFκB- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 2008;**294**:C675–C682.

87 S. Ohnishi, T. Yasuda, S. Kitamura, N. Nagaya. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells* 2007;**25**:1166–1177.

88 P. Fidelis-de-Oliveira, J. P. Werneck-de-Castro, V. Pinho-Ribeiro, *et al.* Soluble factors from multipotent mesenchymal stromal cells have antinecrotic effect on cardiomyocytes *in vitro* and improve cardiac function in infarcted rat hearts. *Cell Transplant* 2012;**21**: 1011–1021.

89 T. L. Yew, Y. T. Hung, H. Y. Li, *et al.* Enhancement of wound healing by human multipotent stromal cell conditioned medium: the paracrine factors and p38 MAPK activation. *Cell Transplant* 2011;**20**:693–706.

90 Z. Gao, Q. Zhang, Y. Han, *et al.* Mesenchymal stromal cell-conditioned medium prevents radiation-induced small intestine injury in mice. *Cytotherapy* 2012;**14**:267–273.

91 C. Y. Wang, H. B. Yang, H. S. Hsu, *et al.* Mesenchymal stem cell-conditioned medium facilitates angiogenesis and fracture healing in diabetic rats. *J Tissue Eng Regen Med* 2012;**6**:559–569.

92 H. J. Hwang, W. Chang, B. W. Song, *et al.* Antiarrhythmic potential of mesenchymal stem cell is modulated by hypoxic environment. *J Am Coll Cardiol* 2012;**60**:1698–1706.

93 Y.-L. Lin, S.-F. Yet, Y.-T. Hsu, *et al.* Mesenchymal stem cells ameliorate atherosclerotic lesions via restoring endothelial function. *Stem Cells Transl Med* 2015;**4**:44–55. doi: 10.5966/sctm .2014-0091.

94 W. L. Grayson, F. Zhao, R. Izadpanah, *et al.* Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol* 2006;**207**:331–339.

95 J. Rehman, D. Traktuev, J. Li, *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;**109**:1292–1298.

96 R. C. Lai, T. S. Chen, S. K. Lim. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* 2011;**6**:481–492.

97 M. A. LaBarge, H. M. Blau. Biological progression from adult bone marrow to mononucleate muscle stem cell to multinucleate muscle fiber in response to injury. *Cell* 2002;**111**:589–601.

98 J. Ankrum, J. M. Karp. Mesenchymal stem cell therapy: two steps forward, one step back. *Trends Mol Med* 2010;**16**:203–209.

99 D. G. Phinney, D. J. Prockop. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* 2007;**25**:2896–2902.

100 N. Wei, S. P. Yu, X. Gu, *et al.* Delayed intranasal delivery of hypoxic-preconditioned bone marrow mesenchymal stem cells enhanced cell homing and therapeutic benefits after ischemic stroke in mice. *Cell Transplant* 2013;**22**:977–991.

101 H. Liu, S. Liu, Y. Li, *et al.* The role of SDF-1–CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. *PLoS ONE* 2012;**7**: e34608.

102 X. Hu, L. Wei, T. M. Taylor, *et al.* Hypoxic preconditioning enhances bone marrow mesenchymal stem cell migration via $K_v2.1$ channel and FAK activation. *Am J Physiol Cell Physiol* 2011;**301**:C362–C372.

103 L. Leroux, B. Descamps, N. F. Tojais, *et al.* Hypoxia preconditioned mesenchymal stem cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther* 2010;**18**:1545–1552.

104 H. Okuyama, B. Krishnamachary, Y. F. Zhou, *et al.* Expression of vascular endothelial growth factor receptor 1 in bone marrow-derived mesenchymal cells is dependent on hypoxia-inducible factor 1. *J Biol Chem* 2006;**281**:15554–15563.

105 C. Busletta, E. Novo, L. Valfre Di Bonzo, *et al.* Dissection of the biphasic nature of hypoxia-induced motogenic action in bone marrow-derived human mesenchymal stem cells. *Stem Cells* 2011;**29**:952–963.

106 G. Lu, H. K. Haider, S. Jiang, M. Ashraf. Sca-$1^+$ stem cell survival and engraftment in the infarcted heart: dual role for preconditioning-induced connexin-43. *Circulation* 2009;**119**:2587–2596.

107 I. Rosova, M. Dao, B. Capoccia, *et al.* Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 2008;**26**:2173–2182.

108 C. C. Huang, D. Y. Chen, H. J. Wei, *et al.* Hypoxia-induced therapeutic neovascularization in a mouse model of an ischemic limb using cell aggregates composed of HUVECs and cbMSCs. *Biomaterials* 2013;**34**:9441–9450.

109 J. M. Hare, J. H. Traverse, T. D. Henry, *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (Prochymal) after acute myocardial infarction. *J Am Coll Cardiol* 2009;**54**:2277–2286.

110 K. Le Blanc, C. Tammik, K. Rosendahl, *et al.* HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;**31**:890–896.

111 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**: 3838–3843.

112 F. Djouad, P. Plence, C. Bony, *et al.* Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;**102**:3837–3844.

113 A. Bartholomew, C. Sturgeon, M. Siatskas, *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;**30**:42–48.

114 K. Le Blanc, I. Rasmusson, B. Sundberg, *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–1441.

115 A. J. Nauta, G. Westerhuis, A. B. Kruisselbrink, *et al.* Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006;**108**:2114–2120.

116 G. M. Spaggiari, A. Capobianco, S. Becchetti, *et al.* Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;**107**:1484–1490.

117 N. Eliopoulos, J. Stagg, L. Lejeune, *et al.* Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* 2005;**106**:4057–4065.

118 T. L. Yew, T. F. Huang, H. L. Ma, *et al.* Scale-up of MSC under hypoxic conditions for allogeneic transplantation and enhancing bony regeneration in a rabbit calvarial defect model. *J Orthop Res* 2012;**30**:1213–1220.

119 T. F. Huang, T. L. Yew, E. R. Chiang, *et al.* Mesenchymal stem cells from a hypoxic culture improve and engraft Achilles tendon repair. *Am J Sports Med* 2013;**41**:1117–1125.

120 W. H. Huang, H. L. Chen, P. H. Huang, *et al.* Hypoxic mesenchymal stem cells engraft and ameliorate limb ischaemia in allogeneic recipients. *Cardiovasc Res* 2014;**101**:266–276.

121 J. Jaussaud, M. Biais, J. Calderon, *et al.* Hypoxia-preconditioned mesenchymal stromal cells improve cardiac function in a swine model of chronic myocardial ischaemia. *Eur J Cardiothorac Surg* 2013;**43**:1050–1057.

CHAPTER 17

# The role of cyclic tensile strain on osteogenesis and angiogenesis in human mesenchymal stem/stromal cells

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## 17.1 Introduction

Mesenchymal stem/stromal cells (MSCs) are a mechanosensitive, promising cell source for cell and tissue engineering therapies. The cells can be isolated from multiple tissues, including bone marrow, adipose tissue, gingiva, skin, uterus, oviduct, and from physiological waste such as urine and menstrual blood [1–6]. As a reservoir cell source residing within fully differentiated tissues, MSCs experience and respond to both chemical and mechanical stimuli in the *in situ* environment. MSCs are highly sensitive to mechanical stimuli in their undifferentiated state. We and others have shown that appropriate mechanical loads can regulate MSC differentiation response *in vitro* [7–13]. A variety of cellular structures and proteins have been implicated as mechanosensors and mechanotransducers in MSCs and will be discussed in subsequent sections of this chapter.

Day-to-day activities and normal physiological processes exert a variety of mechanical loading environments on the human body, with the musculoskeletal tissue bearing some of the highest loads. The crucial role of mechanical loading for healthy bone has long been recognized, particularly in the context of bone loss observed in patients requiring prolonged bed rest and in astronauts exposed to a microgravity environment. Generally, a certain level of mechanical loading is understood to maintain bone homeostasis, as evidenced by increased bone formation in response to regular load-bearing exercise. The cells of the cardiovascular system also undergo extensive mechanical loading. In the aortic blood vessels, smooth muscle and endothelial cells are exposed to cyclic stretch as a result of pulsatile blood flow.

Changes in the mechanical environment have also been found to contribute to the process of skin tissue regeneration during wound healing [14,15]. Multiaxial tension generated during the process of wound healing alters the wound morphology from a circular incision into an elliptical shape, elongated toward the

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direction of greater tensile strain [16]. Tension produced during the wound healing process is also known to induce cell proliferation in skin tissue, with migration of keratinocytes and epidermal cells toward the wound void [17]. Successful generation of engineered tissues that can withstand physiological mechanical environments is critical to the success of tissue replacement therapies in the clinic.

Mechanical loading alone can shape cell and tissue phenotypes. It is therefore critical to understand and elucidate the specific loading modality and magnitude of the forces applied. Specific loading parameters can alter MSC response via alterations in gene expression, type of extracellular matrix (ECM) production, and differentiation into various lineages. Typically, application of mechanical stimulation to cells or tissues *in vitro* is based on attempts to mimic the *in vivo* mechanical environment.

Physical stimuli that affect musculoskeletal tissues include shear stress (often applied as fluid flow), tensile strain, compression, hydrostatic pressure, electrical stimulation, and substrate stiffness. In this chapter we specifically focus on tensile strain, a mechanical loading modality that is relevant to many tissues, including blood vessels, muscle, bone, tendon, and ligament. While tensile forces in the bone may not be as intuitive as in elastic tissues in tendon or muscle, our mechanobiology studies demonstrated that certain magnitudes of tensile strain significantly enhance formation of new bone [10,18–25]. In fully differentiated bone tissue, distraction osteogenesis is a surgical method by which bone is osteotomized and placed under tensile strain to encourage new bone tissue growth, resulting in expanded bone tissue. (An osteotomy is a surgical operation in which a bone is cut to shorten, lengthen, or change its alignment.) Both empirical and computational studies have identified optimal bone formation at tensile strains at a magnitude of approximately 10–12% [24,26]. These distraction osteogenesis analyses formed the basis for culturing human stem/stromal cells under tensile strain for many *in vitro* approaches to osteogenic differentiation. (Distraction osteogenesis is a surgical process used to reconstruct skeletal deformities and lengthen the long bones of the body. A corticotomy is used to fracture the bone into two segments and the two bone ends of the bone are gradually moved apart during the distraction phase, allowing new bone to form in the gap. When the desired or possible length is reached, a consolidation phase follows in which the bone is allowed to keep healing. Distraction osteogenesis has the benefit of simultaneously increasing bone length and the volume of surrounding soft tissues.)

Our group has worked with both adipose-derived and bone-marrow-derived MSCs in both two- (2D) and three-dimensional (3D) cultures. In all cases, application of 10% cyclic tensile strain increased osteogenesis of these cells compared with static culture [10,18–25].

Further, recent microarray studies from our group have shown that 10% cyclic tensile strain increases not only genes indicative of osteogenesis but also those for angiogenic factors. In this chapter the response of MSCs to cyclic tensile strain will be discussed in the context of both osteogenesis and angiogenesis.

## 17.2 Applications of tensile strain: an interpretation from physiological stimuli *in vivo* to bioreactors *in vitro*

As already described, tensile strain is a mechanical stimulus that occurs in multiple types of tissue, including muscle, tendon, ligament, cardiovascular tissues, and during bone formation in distraction osteogenesis. A multitude of bioreactors have been designed for *in vitro* application of tensile strain to tissues or cells. Dense elastic tissue (such as vascular tissue) can be excised and then exposed to tensile strain using an appropriate bioreactor to maintain or enhance its vascular phenotype through emulation of the native mechanical environment [27,28]. Select cells can be seeded onto elastomeric materials such as silicone membranes or embedded in materials such as collagen gels and/or electrospun polymers [29–33]. Biomaterials can be used to modulate the architecture and dimensionality of the culture environment for cells in 2D or 3D culture systems. The material architecture used for 2D culture systems can vary from fibrous to spongy, or even to a rubber structure. The 3D culture material properties can vary even more. However, there are many challenges associated with their use, such as cell-seeding, dispersion, and nutrient perfusion throughout the 3D construct. As a result, 3D culture is frequently performed with gel-like materials (e.g., collagen) in which cells can be thoroughly mixed with the gel in its liquid form. Once the gel polymerizes and solidifies the cells are fully embedded and distributed throughout the construct. Whether cells are seeded on 2D substrates or embedded within 3D materials, tensile strain can be applied in the form of uniaxial or equi-/biaxial strain, dependent upon the bioreactor design.

### 17.2.1 Uniaxial tensile strain

The cells in musculoskeletal tissues are predominantly exposed to uniaxial cyclic tensile strain as a result of normal daily physiological activities. A common bioreactor design for application of uniaxial tensile strain is often composed of a string-shaped elastomeric material as a substrate for cell-adherent (2D) or cell-ingrowth (3D) culture, placed within a chamber containing culture medium, and strained via an actuator (Figure 17.1).

Tissue-mimetic 3D constructs comprised of elastomeric materials can be pulled and retracted from one end, or both ends, during the application of cyclic tensile strain. Actuation can be generated from an actuator grip, a vacuum suction pump, or by bending approaches (e.g., three-point or four-point) [34–36]. (An actuator is a type of motor that is responsible for moving or controlling a mechanism or system; the three- and four-four point bending tests provide values for the modulus of elasticity in bending, flexural stress, flexural strain, and the flexural stress–strain response of the material.)

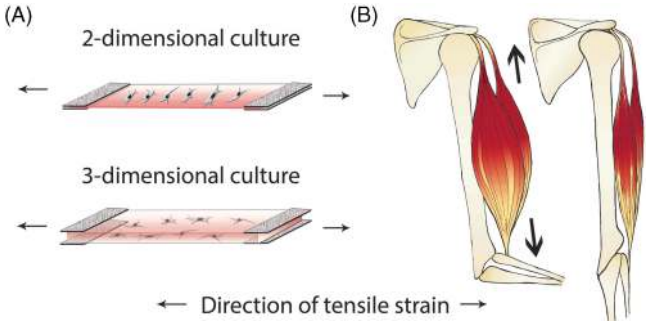



**Figure 17.1** Interpretation of uniaxial tensile strain from physiological forces in musculoskeletal tissue translated to an *in vitro* culture system. (A) Elastomeric material with cell-adherent (2D) and cell ingrowth (3D) systems for *in vitro* uniaxial tensile strain application. (B) Simplified idealized representation of *in vivo* uniaxial tensile strain in musculoskeletal tissue during arm flexure. Arrows show the direction of tensile strain. (See color plate section.)

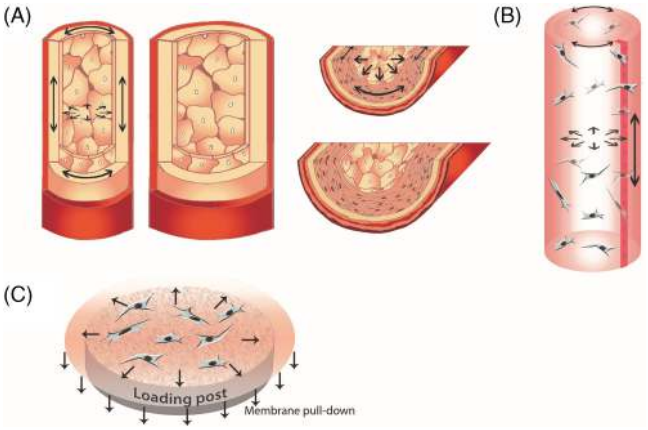



**Figure 17.2** Interpretation of equi-/biaxial tensile strain from physiological forces of an idealized blood vessel translated to an *in vitro* culture system. (A) Expansion of blood vessel and shear stresses from pulsatile blood flow. For a simplified, idealized analysis, smooth muscle cells and endothelial cells in the blood vessel are sometimes modeled as experiencing equi-/biaxial tensile forces during the expansion of blood vessel. (B) A simple 3D *in vitro* culture system to mimic physiological forces in blood vessels. (C) A simplified 2D *in vitro* culture system for the application of equi-/biaxial tensile strain. (See color plate section.)

Such models have been used to understand, apply, and elucidate tensile stimuli in the context of bone, muscle, and tendon tissue as comparable forces, stresses, and strains experienced by the cells *in vivo* (Figure 17.1B).

### 17.2.2 Equi-/biaxial tensile strain

While a uniaxial tensile strain approach simplifies multidirectional tensile stimuli to the cells, it also provides a straightforward mechanical model system to mimic the mechanical environments in skeletal muscle, bone, ligament, and tendon. Equi-/biaxial tensile strain is another loading modality and can be used to better represent mechanical environments during the process of wound healing, expansion and contraction of internal organs by smooth muscle, and in some cases changes in aortic blood vessel diameter during pulsatile blood flow (Figure 17.2A)

In the case of multiaxial tensile strain, an *in vitro* model system commonly used to mimic tensile strain in vascular tissue is a pulsatile flow stretch bioreactor. In such a system the elastomeric biomimetic tissue constructs are formed in a cylindrical shape and then expanded multiaxially by application of pulsatile flow of culture medium through the cylinder [37–39] (Figure 17.2B).

Another simplified system that is widely used employs the use of a soft elastic membrane on a flat surface that is stretched by a vacuum force. While this model does not exactly match the *in vivo* architecture and mechanical environment of a blood vessel (which is exposed to both fluid shear and hoop stresses/strains), it complies to some extent with the localized forces experienced by the cells (Figure 17.2C).

Localized cellular forces often differ from tissue-level forces and must also be considered in the creation and utilization of *in vitro* culture systems. It has been shown that application of 2D equi-/biaxial strain in some systems results in consistent equi-/biaxial strain only in the center area of the circular well, while the edges of the well are predominantly subjected just to uniaxial forces [40,41]. Not unexpectedly, differences in these patterns of localized force affect cell morphology. An investigation of human dermal fibroblasts by Xie *et al.* showed that the application of 7% equiaxial tensile strain at low frequency (1 cycle/min) for 2 days resulted in fibroblastic-shaped cells in the central area and round/polygonal cell shapes at the edge area [41]. In another study with porcine chondrocytes, application of 10% equiaxial tensile strain at 0.5 Hz for 24 h resulted in different cell morphologies: cells in the center area were polygonal in shape (remained a chondrocytic shape) while cells on the edge were spindle shaped and were aligned perpendicular to the radial direction. Similarly, in adipose tissue-derived MSCs (ADSCs) cultured two-dimensionally under 10% cyclic tensile strain on Bioflex (Flexcell, Inc.) plates, we observed reorientation of the cells perpendicular to the axis of uniaxial strain and independent of chemical induction media (i.e., regardless of whether ADSCs were cultured in expansion medium, osteogenic differentiation medium, or adipogenic differentiation medium). Additionally, the application of tensile strain altered the morphology of the ADSCs, causing them to become more elongated and tightly packed, particularly in osteogenically differentiated ADSCs (Figure 17.3).

These changes in cell morphology and alignment occur simultaneously with the alteration of cytoskeletal alignment within the cells. The orientation of microfilaments composed of actin–myosin inside skeletal muscle cells and smooth muscle cells are naturally occurring physiological examples of functional cytoskeletal alignment in response to tensile strain. These mechanical subunits are found to arrange parallel to the direction of tissue tension (Figure 17.4).

In the next section we discuss methods by which cells detect changes in their mechanical environment.

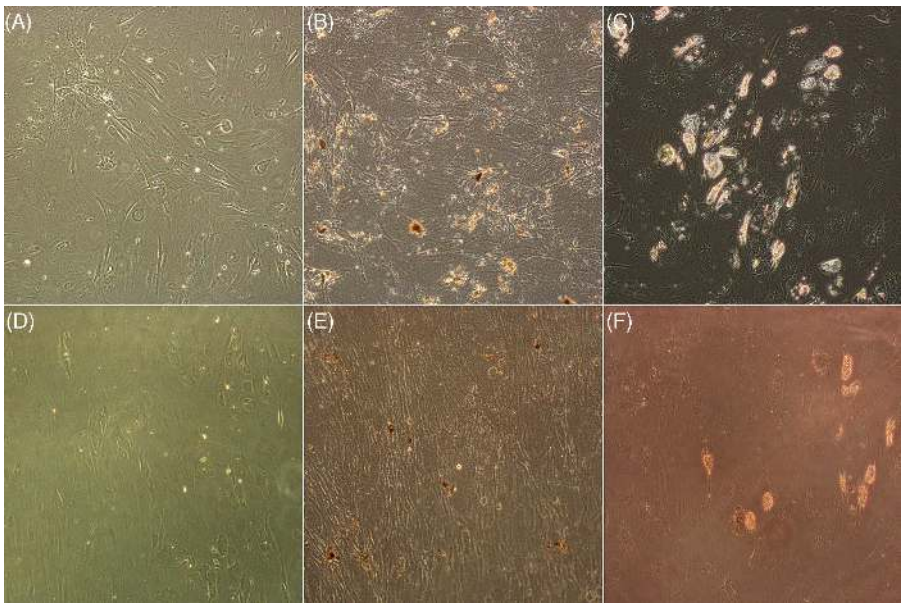



**Figure 17.3** ADSCs cultured for 14 days in expansion medium (a, d), osteogenic induction medium (b, e), or adipogenic induction medium (c, f) under static culture conditions (a–c) or under 10% cyclic tensile strain (d–f) for 4 h/day at a frequency of 1 Hz. The axis of uniaxial strain runs from left to right. (See color plate section.)

## 17.3 Mechanical sensing of mesenchymal stem/stromal cells

MSs express mechanosensitivity to a wide range of mechanical environments, most notably cyclic tensile strain, fluid shear, and substrate stiffness. Typically, integrin binding and cytoskeletal reorganization are thought to be the primary players in MSC mechanosensation, although in recent years the nucleoskeleton, the primary cilium, the glycocalyx and stretch-activated ion channels have been recognized as other important mediators of mechanosensation and mechanotransduction processes.

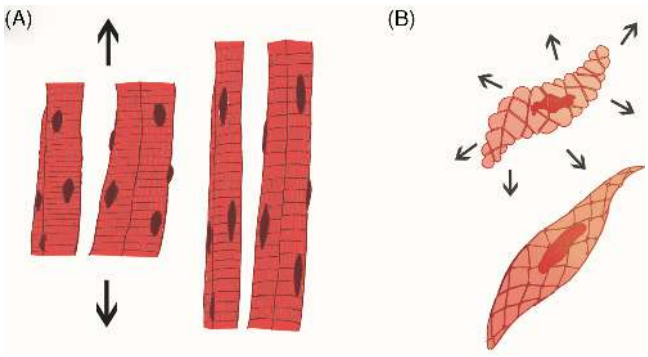



**Figure 17.4** Differences in cell morphology and cytoskeletal alignment between skeletal muscle and smooth muscle. (A) Skeletal muscle cells are elongated with a cylindrical morphology and with actin–myosin microfilament alignment parallel to the uniaxial direction of tissue tension and cell expansion/contraction. (B) Smooth muscle cells are spindle shaped and the actin–myosin microfilaments are aligned in a multidirectional network consistent with multiaxial cell expansion/contraction.

### 17.3.1 Integrins and the cytoskeleton

Integrin and cytoskeletal activity is one of the more thoroughly studied areas of cellular mechanosensation, and their activity is in part responsible for the MSC response to mechanical stimulation. Together, the integrins and cytoskeletal proteins form the foundation of the cellular tensegrity model. The theory of cellular tensegrity proposes a process through which cytoskeletal proteins integrate their function through a balance of global tension coupled with localized compressive forces within the cytoskeleton [42]. More specifically, the actin filaments provide the primary tensional component to the tensegrity model, while the microtubules provide a compression-resistant structural brace in the system. Lastly, intermediate filaments are hypothesized to stabilize the nucleus [42]. In addition to the cytoskeletal pieces of the tensegrity model, the cells must be anchored to the surrounding ECM in order to generate intracellular tension, which is where integrins come in. Integrins provide the protein anchors connecting the cells to the surrounding matrix. Since the initial introduction of the concept of cellular tensegrity, empirical and computational research has confirmed and expanded this model of cellular mechanics [43].

Integrins are heterodimeric transmembrane proteins that bind to specific active-site peptides (such as arginine, glycine, and aspartic acid sites) on the surrounding ECM environment [44]. They provide a platform by which MSCs can transmit and receive mechanical signals through contracting the ECM. The signals transmitted by the integrins are thought to be integrated by focal adhesion kinase (FAK), frequently in the form of focal adhesions comprised of actin bundles that anchor to the intracellular portion of integrin proteins via adaptor proteins such as vinculin [44]. The maturation of focal adhesions allows cells to generate traction forces that in turn allow cells to contract and pull on their surrounding matrix environment. FAK has

been implicated not only as a mechanosensory protein facilitating focal adhesion formation, but also as a regulator of mechanically directed lineage commitment in MSCs. FAK signaling controls cyclic tensile-strain-enhanced collagen-I-induced osteogenic differentiation of human mesenchymal stem/stromal cells [45].

Blocking specific integrins on MSCs has been shown to alter their ability to detect and respond to a given mode of mechanical stimulation or culture environment [46]. Substrate stiffness is one of the most prevalent and direct ways to modulate the mechanics of the culture environment, and this is frequently used to direct MSC differentiation [47]. A study of MSCs and osteogenic differentiation showed that MSCs upregulated FAK during substrate-enhanced osteogenic differentiation and that the $\alpha_2$-integrin in part mediated the enhanced osteogenic response of MSCs on rigid culture substrates [46]. Another study with MSCs cultured on laminin, collagen I, fibronectin, or adult rat cardiac ECM-coated polyacrylamide gels demonstrated that the integrin β1 subunit plays a specific role in substrate-mediated traction forces within the cell [48]. In general, this study reported an increase of MSC β1 integrin expression with increasing substrate rigidity and thus increasing intracellular traction force on all ECM coatings with the exception of adult rat cardiac ECM, which yielded MSCs with unchanged traction forces with increasing substrate stiffness. However, inhibition of β1 integrin binding restored MSC stiffness-mediated behavior with an increase in traction forces, suggesting that β1 integrin may have a specific signaling role in modulating the effect of substrate stiffness, specifically in the mature cardiac ECM [48].

In addition to integrin-mediated mechanosensing mechanisms, the cytoskeletal proteins play an important role in this process. In MSCs cultured in agarose gels, vimentin, an intermediate filament protein, was identified as a mechanosensitive protein in MSCs exposed to cyclic hydrostatic pressure in chondrogenic differentiation medium [49]. Furthermore, this study noted the importance of pericellular matrix formation and its mechanical properties, which in turn regulate MSC mechanosensation in a 3D gel environment.

Studies in MSCs that disrupt cytoskeletal reorganization in response to mechanical stimulation and/or disrupt focal adhesion formation provide strong evidence of this mechanosensation mechanism. This has been shown directly in MSCs by Müller *et al.* following treatment with drugs that depolymerize or stabilize actin filaments [50]. Using magnetic beads, a force was applied directly to MSCs via β1-integrin to MSCs with depolymerized or stabilized actin cytoskeletons. MSCs with disrupted cytoskeletal organization exhibited suppressed extracellular signal-regulated kinase (ERK) activation in response to mechanical stimulation compared with MSCs with intact cytoskeletal structures. Moreover, characteristic mechanically enhanced osteogenic differentiation was abrogated in MSCs with cytoskeletal inhibition, suggesting that cytoskeletal disruption also plays a role in the differentiation capabilities of MSCs [50]. Cytoskeletal organization plays a critical role in cell morphology, which is directly linked to the physiological function of a particular cell lineage. When the actin cytoskeleton is disrupted, MSCs change or lose their proclivity to commitment to specific cell phenotypes. Modulating cytoskeletal tension induced by the surrounding mechanical environment has also been shown to influence mechanically induced MSC lineage specification processes [51].

Interestingly, when exposed to cyclic tensile strain in 2D culture, MSCs typically undergo actin cytoskeletal reorganization and the cells align perpendicularly to the direction of strain to minimize stress and disruption of focal adhesions [52].

We have observed that, in 3D culture, MSCs present a differential response with their actin cytoskeleton aligning along the direction of strain, suggesting that uniaxial tensile strain applied to MSCs cultured three-dimensionally yields a different physiological effect to that of MSCs cultured two-dimensionally.

### 17.3.2 The nucleoskeleton and lamins

Nuclear lamins, also known as Class V intermediate filaments and not to be confused with the laminins of the ECM, are fibrous proteins providing structural function and transcriptional regulation in the cell nucleus. Nuclear lamins interact with membrane-associated proteins to form the nuclear lamina on the interior of the nuclear envelope. They are involved in the breakdown and reformation of the nuclear envelope during mitosis as well as the positioning of nuclear pores.

Recent studies on nuclear membrane proteins such as lamins have revealed a critical mechanosensing function of the nucleoskeleton. A study by Swift *et al.* showed that lamin A expression scales with stiffness in MSCs [53]. On a stiff substrate (40 kPa) their nuclei appeared flattened, spread and with a smooth surface, corresponding to increased lamin A expression. Conversely, MSCs cultured on a soft substrate (0.3 kPa) expressed wrinkled, rounded nuclei and decreased nuclear lamin A expression. Additionally, knockdown of lamin A expression lowered the effective nuclear stiffness and enhanced the effects of soft substrate-directed adipogenesis, whereas overexpression of lamin A enhanced substrate-directed osteogenesis on a stiff substrate. When lamin A is knocked down, MSCs have a limited capacity to generate cytoskeletal tension and reorganization, suggesting that the nuclear skeletal proteins are required for MSC mechanosensation. Further, another study from the same group reported that lamin-A and -C knockdown in MSCs conferred downregulation in expression of actomyosin genes, implicating an interplay between the nucleoskeleton and the cytoskeleton [54]. Recent reports on lamins and the nucleoskeleton are beginning to elucidate a new component of MSC mechanosensation as well as providing empirical support for the tensegrity model.

### 17.3.3 Primary cilia

In humans, primary cilia are found on nearly every cell in the body. In comparison with motile cilia, nonmotile (or primary)

cilia usually occur at a frequency of one per cell. Nearly all mammalian cells have a single nonmotile primary cilium. Recent findings regarding its physiological roles in chemical sensation, signal transduction, and control of cell growth have led to an acknowledgement of its importance in cell function. The primary cilium is now known to play an important role in the function of many human organs. The current scientific understanding of primary cilia views them as sensory cellular antennae that coordinate a large number of cellular signaling pathways, sometimes coupling the signaling to ciliary motility or alternatively to cell division and differentiation. A scanning electron microscopic image of primary cilia in the lungs is shown in Figure 17.5.

Primary cilia have also been identified as an additional mechanosensing structure in a variety of cell types, including MSCs [55]. In bone cells the primary cilium is thought to deflect under fluid shear and promote a prototypical osteogenic cell response to this mechanical stimulation. However, in the absence of a prominent primary cilium structure, osteogenic cells lose their sensitivity to fluid shear stimulation [56]. Additionally, in tenocytes, primary cilia modify their length in response to a cyclic tensile strain loading environment [57].

Primary cilia on MSCs exhibit a similar response to osteoblasts under oscillatory fluid flow, acting as mechanotransducers of fluid shear. More specifically, when primary cilia expression was disrupted through small interfering RNA (siRNA) knockdown of Polaris, a cilia-associated structural protein, MSCs exhibited a loss of their characteristic osteogenic gene upregulation in response to oscillatory fluid flow [55]. The MSC primary cilia not only mediated mechanically induced osteogenic differentiation but also regulated mechanically enhanced proliferation. Fluid shear increased the proliferation activity of MSCs, and this was more pronounced in MSCs devoid of Polaris siRNA [55].

In addition to cilia mechanosensitivity to active sources of mechanical stimulation, topographical architecture has also been shown to dictate primary cilia orientation and structure expression, suggesting that the cilium structure has some sort of intrinsic mechanosensitivity to the culture substrate [58]. MSCs cultured on grooved substrates exhibited cellular and ciliary alignment along the direction of the grooved surface. Further, the primary cilium structure was on average longer on MSCs on the grooved surface than that of MSCs cultured on a flat surface. Topography-directed changes in cilia expression were Rock/Rho dependent, suggesting that actin organization is involved in cilia expression and extension. Additionally, this study highlighted an important relationship between Wnt signaling and changes in cilia structure, which may have a mechanosensitive component.

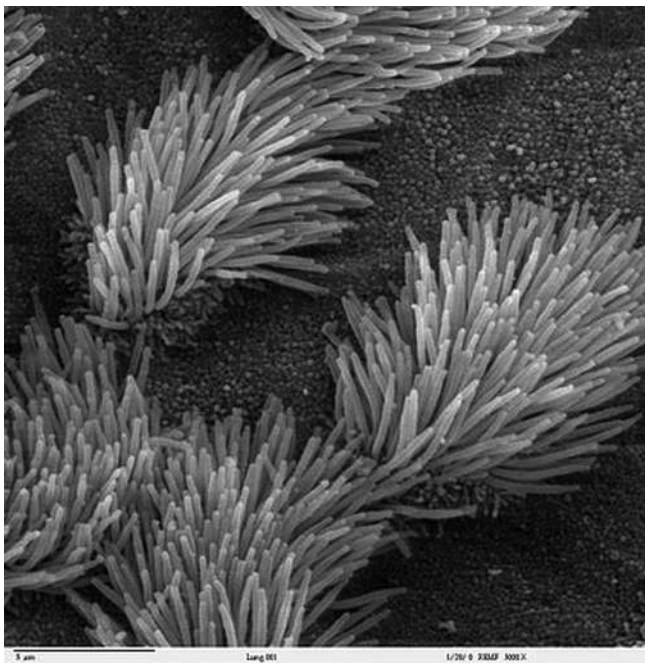


**Figure 17.5** Scanning electron microscope image of lung trachea epithelium showing primary cilia on tracheal epithelial cells. There are both ciliated and non-ciliated cells in this epithelium. Note the difference in size between the cilia and the microvilli (on the non-ciliated cell surface). *Source:* http://commons.wikimedia.org/wiki/File:Bronchiolar_epithelium_3_-_SEM.jpg#/media/File:Bronchiolar_epithelium_3_-_SEM.jpg.

### 17.3.4 Stretch-activated calcium channels

Stretch-activated ion channels are another mechanism by which MSCs can sense the surrounding environment. The physical application of strain to the membrane of a cell can open or activate particular ion channels, altering the flow of ions between the intracellular and extracellular spaces. MSCs seeded onto 3D scaffolds exposed to 10% cyclic tensile strain and cultured in chondrogenic culture medium displayed an increase in glycosaminoglycan (GAG) synthesis, characteristic of chondrogenic differentiation. This GAG synthesis was higher than GAG synthesis in MSCs cultured in chondrogenic medium under static culture. However, when stretch-activated ion channels were blocked with gadolinium chloride, GAG synthesis in strained MSCs was significantly reduced, suggesting that stretch-activated ion channels likely regulate mechanically induced chondrogenic differentiation of MSCs [59]. The reduction in GAG synthesis with blocked ion channels was significantly lower than that in untreated MSCs cultured under tensile strain. However, they were still higher than GAG levels on chondrogenically differentiated MSCs, thus suggesting that stretch-activated ion channels are not the only mechanosensors in this process.

### 17.3.5 The glycocalyx

The glycocalyx is a glycoprotein polysaccharide covering that surrounds the cell membranes of some bacteria, epithelia, and other cells. Most animal epithelial cells have a fuzz-like coat on the external surface of their plasma membranes. This coating consists of several carbohydrate moieties of membrane glycolipids and glycoproteins, which serve as backbone molecules for

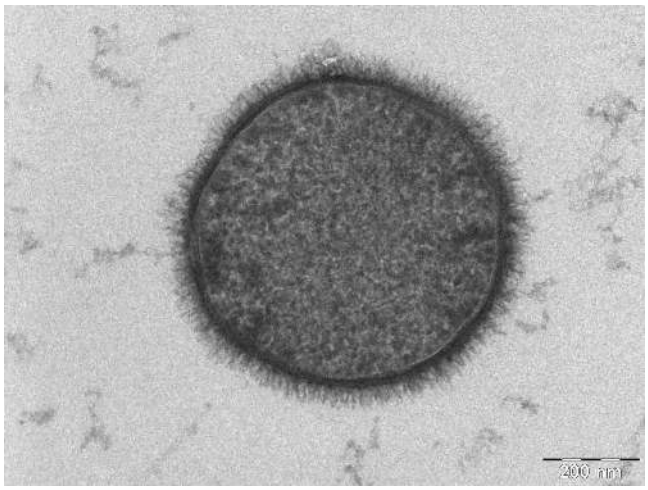



**Figure 17.6** Transmission electromicrograph of a *Bacillus subtilis* bacterium with the hair-like glycocalyx visible surrounding the cell membrane (scale bar: 200 nm). *Source:* http://commons.wikimedia.org/wiki/File:Bacillus_subtilis.jpg#/media/File:Bacillus_subtilis.jpg.

support. Generally, the carbohydrate portion of the glycolipids found on the surface of plasma membranes helps these molecules contribute to cell–cell recognition, communication, and intracellular adhesion. The glycocalyx is a type of identifier that the body uses to distinguish between its own healthy cells and transplanted tissues, diseased cells, or invading organisms. Included in the glycocalyx are cell-adhesion molecules that enable cells to adhere to each other and guide the movement of cells during embryonic development. The glycocalyx plays a major role in regulation of endothelial vascular tissue, including the modulation of red blood cell volume in capillaries (Figure 17.6).

The glycocalyx frequently forms on the apical cell membrane surface of many cell types in 2D culture, and some hypothesize that this structure is present in MSCs. Perturbations in the glycocalyx as a result of mechanical stimulation are generally thought to transduce mechanical signals to the cell, and this structure has been frequently implicated in endothelial cell mechanotransduction [60]. There is some evidence that a glycocalyx is present in MSCs derived from the umbilical cord, and the glycocalyx is potentially another mechanosensory structure of MSCs [61]. However, mechanisms downstream of the glycocalyx remain unclear.

## 17.4 The molecular response of mesenchymal stem/stromal cells to cyclic tensile strain

The fundamental responses of MSCs to changes in the mechanical environment often minimize cellular injury and/or promote physiological functions in the body. The MSC response to mechanical stimuli integrates with tissue function. However, the mechanisms underlying the MSC response are complex and somewhat elusive. For the purposes of this chapter the MSC response to mechanical loading will be divided into two major categories (Figure 17.7).

The first one encompasses MSC response with respect to changes within the cells themselves or via modification of their surrounding ECM. Such adaptations to the mechanical environment include reorganization of the cytoskeleton, changes in cell morphology, cell differentiation, rearrangement of the overall tissue structure by increases in cell number, and production of specific types of ECM and/or release of proteases to degrade or remodel the existing ECM.

The second category involves the MSC secretome and the autocrine and paracrine effects of MSCs on other cells or tissues. The active proteins and molecules secreted by MSCs induce responses from other tissues throughout the body, including cell proliferation, cell migration to the site, and vascular ingrowth.

In the context of bone, homeostasis requires dynamic tissue remodeling with continuous changes of bone structure via bone formation and bone resorption. This remodeling process is performed by osteoblasts and osteoclasts. While osteoblasts are bone-forming cells derived from the mesenchymal stem cell lineage, osteoclasts are derived from scavenger macrophages in the hematopoietic stem cell lineage. It has been shown that osteoblasts can be induced to produce the receptor activator of nuclear factor kappa B ligand (RANKL), a factor that stimulates the process of bone resorption by osteoclasts [62–64]. In *in vitro* co-culture experiments it was shown that tensile-strain-induced secretion of RANKL by osteoblasts further stimulated the

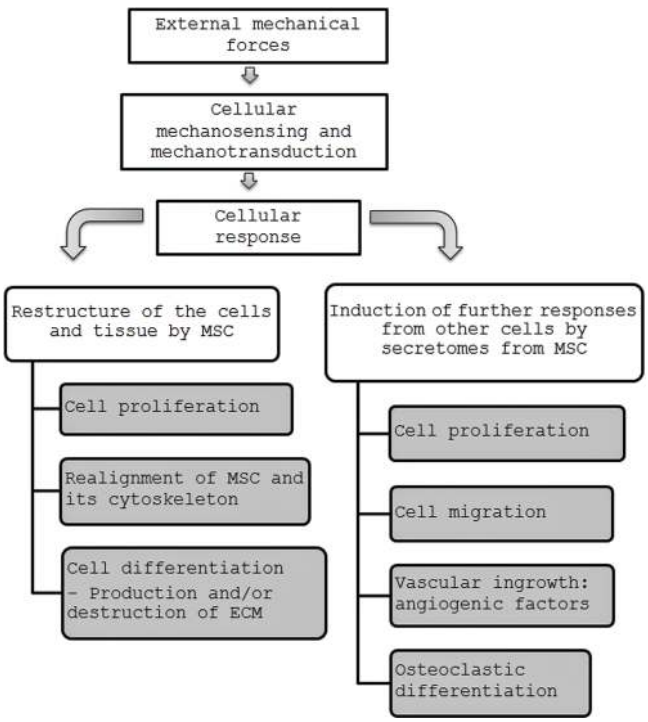



**Figure 17.7** Categorized responses of MSCs to cyclic tensile strain reviewed in this chapter.

production of tartrate-resistant acid phosphatase by murine macrophages [65].

The ability of MSCs to differentiate down an osteogenic pathway both *in vivo* and *in vitro* has been extensively reported [66–70]. *In vivo*, the mesenchymal *stem* cell niche is most commonly thought to reside in the bone marrow. However, the progeny of mesenchymal *stem* cells, known usually as mesenchymal *stromal* cells, can be found in all vascularized tissues, since mesenchymal stromal cells form part of blood vessel endothelium and are pericytes. Mesenchymal *stromal* cells have been isolated from bone, olfactory mucosa, endometrium, adipose tissue, dermal tissue, and many other tissues and can also differentiate down the osteogenic pathway [71–74]. Our previous work showed that uniaxial cyclic tensile strain at a magnitude of 10% induced osteogenesis of human bone-marrow-derived MSCs with calcium deposition, the expression of the bone marker genes bone morphogenetic protein-2 (BMP-2) and osteopontin, as well as the secretion of the inflammatory cytokines interleukin (IL)-6 and IL-8 [23,25,75,76]. Further, microarray analyses showed that signaling pathways regulated by 10% uniaxial cyclic tensile strain in human MSCs and human ADSCs were associated not only with osteogenesis but also with cell proliferation, migration, proinflammatory cytokines, and angiogenesis [19,20]. In the following section, further details of the cellular and molecular responses of MSCs to cyclic tensile strain will be discussed. Tensile-strain-induced cell proliferation, osteogenic differentiation to promote bone formation, and MSC signals that initiate vascular ingrowth or osteoclastogenesis for bone remodeling are evaluated while also considering MSCs as a candidate for therapeutic applications in bone regeneration and bone tissue engineering.

### 17.4.1 Restructuring of mesenchymal stem/stromal cells and the surrounding extracellular matrix by mesenchymal stem/stromal cells in response to cyclic tensile strain

As discussed previously, MSCs change their morphology and surrounding ECM in response to chemical and mechanical environments. In this section, the changes by MSCs in response to cyclic tensile strain will be specifically explored in the context of bone tissue regeneration, including increases in MSC proliferation, changes in cell fates with rearrangement of the cytoskeleton, changes in cell morphology, osteogenic lineage specification, and the secretion of new ECM or enzymes to remodel the existing ECM.

#### 17.4.1.1 Increases in cell proliferation by cyclic tensile strain

An important characteristic of MSCs as a cell reservoir in vascularized tissues is their proliferative ability, which is critical to their role in replacing worn-out or injured tissues. Mesenchymal *stromal* cells can be extensively expanded *in vitro* and maintain their characteristics during long-term cryopreservation, especially if handled by reducing the use of enzymatic digestion [76–78].

Multiple pathways have been shown to be involved in the self-renewal and proliferation of stem cells and their progeny. Most are cell cycle regulatory proteins/antiapoptotic proteins, transcription factors, microRNA regulators, and signal transducers [79–83]. A knockdown and overexpression study of the transcription factors OCT4 and NANOG demonstrated their role in maintaining self-renewal in MSCs [84]. The known regulatory signaling pathways in self-renewal and proliferation of MSCs include transforming growth factor beta (TGF-β), Wnt/BMP, intracellular PI3K-AKT, and MAPK signaling [85–96]. An androgen receptor knockout study of MSCs showed an increased self-renewal ability with the activation of ERK/Akt signaling through epidermal growth factor [94]. Enhanced cell survival and self-renewal of pluripotent stem cells by doxycycline was found to be mediated by direct activation of a PI3K–AKT intracellular signal [95].

Increases in cell proliferation induced by certain magnitudes and duration of cyclic tensile strain have been demonstrated in multiple systems [34,96,97]. A study of rat MSCs found that 4% and 8% equiaxial tensile strain significantly increased rat MSC proliferation [98]. Uniaxial tensile strains at different magnitudes at 0%, 5%, or 10% and different durations ranging from 1 h, 2 h, or 4 h also resulted in increased proliferation of human MSCs in response to uniaxial tensile strain, with maximum proliferation occurring with the application of 10% tensile strain for 4 h [99]. Microarray analysis on the effect of 10% cyclic tensile strain applied for 4 h/day for 14 days on human MSCs isolated from osteoporotic donors showed a higher total RNA yield and modulation of 32 genes involved in cell proliferation. These included upregulation of T-box 3, phosphoinositide-3-kinase, phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta (PIK3CD), insulin-like growth factor 1 receptor, plasminogen activator, urokinase-type plasminogen activator receptor (PLAUR), vascular endothelial growth factor (VEGF) A, PDZ and LIM domain 4 (PDLIM4), and melanoma cell adhesion molecule. There was downregulation of six genes, including paired related homeobox 1, frizzled class receptor 8, and latent transforming growth factor beta binding protein 2 [19].

#### 17.4.1.2 Restructuring of mesenchymal stem/stromal cells by cyclic tensile strain

During the process of cell differentiation and maturation, progenitor cells go through major changes in cell structure with rearrangement of the cytoskeleton and restructuring of cell shape. Cytoskeletal tension has been shown to determine the lineage commitment of human MSCs. Active expression of RhoA allows cells to generate tension, which is required for osteogenesis by differentiated MSCs, while inhibition of RhoA function has been found to decrease myosin-generated cytoskeletal tension, resulting in adipogenesis of MSCs [100]. When cells are exposed to tensile strain, they adapt to this mechanical stimulus with changes in cytoskeletal arrangement and cell shape. Short-term

2D studies of application of tensile strain on endothelial cells showed increases in stress fibers as assessed by parallel arrays of actin filaments with reorientation perpendicular to the direction of strain [101]. Exposure of MSCs to 15% strain for 2 h induced F-actin reorganization perpendicular to the axis of mechanical strain [99]. However, opposite results were found when MSCs were cultured in a 3D scaffold, in which MSCs aligned parallel to the direction of tensile strain [102,103]. These opposite cell-alignment behaviors in 2D and 3D culture systems are likely a result of substrate realignment having a greater influence than the application of uniaxial tensile strain, since cells in 3D culture are highly influenced by contact guidance by the ECM fibers, as is also found in native ligament and muscle tissues [104–107]. Our studies on MSCs cultured in 3D collagen gels also showed that application of uniaxial cyclic tensile strain influenced cells to reorientate parallel to the direction of tensile strain [75,108].

#### 17.4.1.3 Induced osteogenic differentiation of mesenchymal stem/stromal cells in response to cyclic tensile strain: cellular signaling responses and extracellular matrix production/remodeling

Specific magnitudes of tensile strain have been established as mechanical stimuli that can induce osteogenesis from *in vivo* analyses during the process of new tissue formation in distraction osteogenesis to the *in vitro* application of cyclic tensile strain directly to the cells [24,26,109–111]. Many bone markers have been detected as osteogenesis was promoted and increased in response to cyclic tensile strain applied to MSCs, including RUNX2, also known as core-binding factor alpha 1, alkaline phosphatase, collagen type I, osteocalcin, and BMP-2 [25,109]. Cyclic tensile strain alone, however, has not been reported as a factor that induces MSCs to attain the final stage of osteogenic differentiation and deposit calcium phosphate without additional chemical induction *in vitro*. A combination of mechanical loading with chemically induced osteogenic media, however, significantly increased calcium deposition by MSCs both in 2D and 3D cultures relative to unloaded MSCs maintained in osteogenic induction medium alone [20,76,112].

As many genes are used as bone marker genes when determining the effects of cyclic tensile strain on MSCs, it has been assumed that increased osteogenesis by MSCs through mechanical loading includes those genes involved in the signaling pathways that induce osteogenesis by chemical stimuli. Three major signaling pathways that have been identified as associated with osteogenesis of human MSCs by chemical induction and by 10% cyclic tensile strain are Wnt, BMP, and phosphatidylinositide 3-kinases (PIK signaling) [19,20]. Increased production of ECM proteins by MSCs in response to cyclic tensile strain includes collagen I, elastin, and tenascin C [36,113–115].

Using microarray analyses we have also identified other genes that may play a role in enhanced osteogenesis of MSCs in response to cyclic tensile strain, including jun D proto-oncogene, PLAUR, PIK3CD, wingless-type mouse mammary tumor virus integration site family member 5B, and PDLIM4 [19,20]. Further studies are required to fully understand the mechanistic role of these genes.

### 17.4.2 Mesenchymal stem/stromal cell secretomes that induce further responses from other cells

MSCs have shown their ability to generate various secretomes in response to changes in the mechanical environment or during a chemically induced differentiation process [116–118]. Proteomic analysis of secretomes from MSCs isolated from adipose tissue showed 51 proteins that were differentially expressed during the course of a 4-week osteogenic differentiation protocol [119]. Work by Ando *et al.* reported that the secretomes released by cultured human MSCs into the culture medium accelerated the process of distraction osteogenesis in an *in vivo* mouse model [120]. Secretions of MSCs into the culture medium have also been hypothesized as a potential intravesical therapy for interstitial cystitis since it contains antiapoptotic, anti-inflammatory, angiogenic, and immunosuppressive properties [121]. Lovoie and Rosu-Myles have categorized MSC secretomes by their functional roles in immune and inflammatory modulation and paracrine effects on tissue repair [122].

The process of bone healing has been known to involve multiple angiogenic factors, resulting in the formation of new blood vessels in the fracture callus. Angiogenic factors found at the fracture site during regeneration of bone tissue include VEGF, placental growth factor, fibroblast growth factor (FGF), and matrix metalloproteinases (MMPs) [123]. Interestingly, implantation of MSCs or application of factors released by MSCs to bone defect sites resulted in recruitment of macrophages and endothelial cells to the injured sites and enhanced the process of recovery [124,125]. Similarly, an investigation of skin wound healing using the scratch assay showed that the secretomes produced by *in vitro* culture of human MSCs accelerated the wound healing process by accelerating cell migration to, but not cell proliferation at, the wound site [126]. Chemokines and cytokines released from human MSCs include TGF-β1, IL-6, IL-8, chemokine (C–C motif) ligand 2, chemokine (C–C motif) ligand 5, collagen type I, fibronectin, osteonectin (also known as secreted protein, acidic, cysteine-rich), and insulin-like growth factor binding protein [126] (and see Chapters 18, 19, 22, and 23). Further, a study of menstrual-blood-derived MSCs that were co-cultured with monocytes showed that MSCs suppressed maturation of monocyte-derived dendritic cells through secretion of IL-6 and IL-10 [127].

As described earlier, in a co-culture study of osteoblasts and macrophages exposed to tensile strain, it was shown that osteoclastic differentiation of macrophages occurred [65]. In an *in vitro* study of MSCs we showed that the expression of the proinflammatory cytokines IL-6 and IL-8 increased in response to 10% and 12% uniaxial cyclic tensile strain [75]. We then performed a microarray analysis of human ADSCs exposed to 10% cyclic tensile strain for 4 h/day for 14 days and found upregulation of the proinflammatory cytokine regulators IL-1

receptor antagonist, suppressor of cytokine signaling (SOSC) 3, and the angiogenic factors FGF-2, MMP-2, and VEGFA. SOCS3 is a protein that in humans is encoded by the *SOCS3* gene. This gene encodes a member of the signal transducer and activator of transcription (STAT)-induced STAT inhibitor (SSI) (also known as SOCS) family. SSI family members are cytokine-inducible negative regulators of cytokine signaling.

## 17.5 Summary

In summary, MSCs hold considerable potential for use in regenerative medicine applications as indicated by their ability to respond to both chemical and mechanical induction. The MSC response to cyclic tensile strain is of particular interest as this mechanical stimulus appears to induce an MSC response that results in maintenance of physiological function in musculoskeletal and cardiovascular tissue. When appropriate mechanical loading is applied, such as the specific cyclic tensile strains discussed in this chapter, various mechanotransduction mechanisms lead to an MSC response that includes adaptation of the MSCs themselves and adaptations to both the ECM and other types of cells. MSCs proliferate, differentiate, and secrete signaling molecules that further induce migration of other cells for vascular ingrowth and macrophage differentiation to the osteoclastic lineage. While many studies have reported some similarities in increased MSC osteogenesis by chemical and mechanical induction, many genes that have been found to respond during increased osteogenesis of MSCs by cyclic tensile strain are unique and should be explored further.

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## References

1 Zhang Y, McNeill E, Tian H, *et al.* Urine derived cells are a potential source for urological tissue reconstruction. *J Urol* 2008;**180** (5): 2226–2233.
2 Meng X, Ichim TE, Zhong J, *et al.* Endometrial regenerative cells: a novel stem cell population. *J Transl Med* 2007;**5**:57.
3 Estes BT, Diekman BO, Gimble JM, Guilak F. Isolation of adipose-derived stem cells and their induction to a chondrogenic phenotype. *Nat Protoc* 2010;**5** (7):1294–1311.
4 Lukacs RU, Goldstein AS, Lawson DA, *et al.* Isolation, cultivation and characterization of adult murine prostate stem cells. *Nat Protoc* 2010;**5** (4):702–713.
5 Soleimani M, Nadri S. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. *Nat Protoc* 2009;**4** (1):102–106.
6 Bernacki SH, Wall ME, Loboa EG. Isolation of human mesenchymal stem cells from bone and adipose tissue. *Methods Cell Biol* 2008;**86**:257–278.
7 Rui YF, Lui PPY, Ni M, *et al.* Mechanical loading increased BMP-2 expression which promoted osteogenic differentiation of tendon-derived stem cells. *J Orthop Res* 2011;**29** (3):390–396.
8 Sen B, Xie Z, Case N, *et al.* Mechanical strain inhibits adipogenesis in mesenchymal stem cells by stimulating a durable β-catenin signal. *Endocrinology* 2008;**149** (12):6065.
9 Wall ME, Rachlin A, Otey CA, Loboa EG. Human adipose-derived adult stem cells upregulate palladin during osteogenesis and in response to cyclic tensile strain. *Am J Physiol Cell Physiol* 2007;**293** (5):C1532–C1538.
10 Hanson AD, Marvel SW, Bernacki SH, *et al.* Osteogenic effects of rest inserted and continuous cyclic tensile strain on hASC lines with disparate osteodifferentiation capabilities. *Ann Biomed Eng* 2009; **37** (5):955–965.
11 Finger AR, Sargent CY, Dulaney KO, *et al.* Differential effects on messenger ribonucleic acid expression by bone marrow-derived human mesenchymal stem cells seeded in agarose constructs due to ramped and steady applications of cyclic hydrostatic pressure. *Tissue Eng* 2007;**13** (6):1151–1158.
12 Angele P, Yoo J, Smith C, *et al.* Cyclic hydrostatic pressure enhances the chondrogenic phenotype of human mesenchymal progenitor cells differentiated *in vitro*. *J Orthop Res* 2003;**21** (3):451–457.
13 McCullen SD, McQuilling JP, Grossfeld RM, *et al.* Application of low-frequency alternating current electric fields via interdigitated electrodes: effects on cellular viability, cytoplasmic calcium, and osteogenic differentiation of human adipose-derived stem cells. *Tissue Eng Part C Methods* 2010;**16** (6):425–454.
14 Watts GT. Wound shape and tissue tension in healing. *Br J Surg* 1960;**47** (205):555–561.
15 Ksander GA, Vistnes LM, Rose EH. Excisional wound biomechanics, skin tension lines, and elastic contraction. *Plast Reconstr Surg* 1977;**59** (3):398–406.
16 Langer K. On the anatomy and physiology of the skin: I. The cleavability of the cutis. *Br J Plast Surg* 1978;**31** (1):3–8.
17 Evans ND, Oreffo RO, Healy E, *et al.* Epithelial mechanobiology, skin wound healing, and the stem cell niche. *J Mech Behav Biomed Mater* 2013;**28**:397–409.
18 Mathieu PS, Bodle JC, Loboa EG. Primary cilium mechanotransduction of tensile strain in 3D culture: finite element analyses of strain amplification caused by tensile strain applied to a primary cilium embedded in a collagen matrix. *J Biomech* 2014;**47** (9): 2211–2217.
19 Charoenpanich A, Wall ME, Tucker CJ, *et al.* Cyclic tensile strain enhances osteogenesis and angiogenesis in mesenchymal stem cells from osteoporotic donors. *Tissue Eng Part A* 2013;**20**(1–2):67–78.
20 Charoenpanich A, Wall ME, Tucker CJ, *et al.* Microarray analysis of human adipose-derived stem cells in three-dimensional collagen culture: osteogenesis inhibits bone morphogenic protein and Wnt signaling pathways, and cyclic tensile strain causes upregulation of

proinflammatory cytokine regulators and angiogenic factors. *Tissue Eng Part A* 2011;**17**(21–22):2615–2627.
21 Sumanasinghe RD, Pfeiler TW, Monteiro-Riviere NA, Loboa EG. Expression of proinflammatory cytokines by human mesenchymal stem cells in response to cyclic tensile strain. *J Cell Physiol* 2009;**219**:77–83.
22 Rouse JG, Haslauer CM, Loboa EG, Monteiro-Riviere NA. Cyclic tensile strain increases interactions between human epidermal keratinocytes and quantum dot nanoparticles. *Toxicol In Vitro* 2008;**22** (2):491–497.
23 Wall ME, Rachlin A, Otey CA, Loboa EG. Human adipose-derived adult stem cells upregulate palladin during osteogenesis and in response to cyclic tensile strain. *Am J Physiol Cell Physiol* 2007;**293** (5):C1532–C1538.
24 Loboa EG, Fang TD, Parker DW, *et al.* Mechanobiology of mandibular distraction osteogenesis: finite element analyses with a rat model. *J Orthop Res* 2005;**23** (3):663–670.
25 Sumanasinghe RD, Bernacki SH, Loboa EG. Osteogenic differentiation of human mesenchymal stem cells in collagen matrices: effect of uniaxial cyclic tensile strain on bone morphogenetic protein (BMP-2) mRNA expression. *Tissue Eng* 2006;**12** (12):3459–3465.
26 Loboa EG, Fang TD, Warren SM, *et al.* Mechanobiology of mandibular distraction osteogenesis: experimental analyses with a rat model. *Bone* 2004;**34** (2):336–343.
27 Chowdhury B, David AL, Thrasivoulou C, *et al.* Tensile strain increased COX-2 expression and PGE2 release leading to weakening of the human amniotic membrane. *Placenta* 2014;**35** (12): 1057–1064.
28 Lacerda CM, MacLea HB, Kisiday JD, Orton EC. Static and cyclic tensile strain induce myxomatous effector proteins and serotonin in canine mitral valves. *J Vet Cardiol* 2012;**14** (1):223–230.
29 Jeong SI, Kim SY, Cho SK, *et al.* Tissue-engineered vascular grafts composed of marine collagen and PLGA fibers using pulsatile perfusion bioreactors. *Biomaterials* 2007;**28** (6):1115–1122.
30 Wu S, Johansson J, Damdimopoulou P, *et al.* Spider silk for xeno-free long-term self-renewal and differentiation of human pluripotent stem cells. *Biomaterials* 2014;**35** (30):8496–8502.
31 McCullen S, Zhu Y, Bernacki S, *et al.* Electrospun composite poly(L-lactic acid)/tricalcium phosphate scaffolds induce proliferation and osteogenic differentiation of human adipose-derived stem cells. *Biomed Mater* 2009;**4**:035002.
32 Moretti M, Prina-Mello A, Reid A, *et al.* Endothelial cell alignment on cyclically-stretched silicone surfaces. *J Mater Sci Mater Med* 2004;**15** (10):1159–1164.
33 Zhuang J, Yamada KA, Saffitz JE, Kleber AG. Pulsatile stretch remodels cell-to-cell communication in cultured myocytes. *Circ Res* 2000;**87** (4):316–322.
34 Wang Y, Li Y, Fan X, *et al.* Early proliferation alteration and differential gene expression in human periodontal ligament cells subjected to cyclic tensile stress. *Arch Oral Biol* 2011;**56** (2): 177–186.
35 Qi MC, Hu J, Zou SJ, *et al.* Mechanical strain induces osteogenic differentiation: Cbfa1 and Ets-1 expression in stretched rat mesenchymal stem cells. *Int J Oral Maxillofac Surg* 2008;**37** (5):453–458.
36 Baker BM, Shah RP, Huang AH, Mauck RL. Dynamic tensile loading improves the functional properties of mesenchymal stem cell-laden nanofiber-based fibrocartilage. *Tissue Eng Part A* 2011;**17** (9–10):1445–1455.
37 Peng X, Recchia FA, Byrne BJ, *et al. In vitro* system to study realistic pulsatile flow and stretch signaling in cultured vascular cells. *Am J Physiol Cell Physiol* 2000;**279** (3):C797–C805.
38 Sodian R, Lemke T, Loebe M, *et al.* New pulsatile bioreactor for fabrication of tissue-engineered patches. *J Biomed Mater Res* 2001;**58** (4):401–405.
39 Chan-Park MB, Shen JY, Cao Y, *et al.* Biomimetic control of vascular smooth muscle cell morphology and phenotype for functional tissue-engineered small-diameter blood vessels. *J Biomed Mater Res Part A* 2009;**88** (4):1104–1121.
40 Huang J, Ballou L, Hasty K. Cyclic equibiaxial tensile strain induces both anabolic and catabolic responses in articular chondrocytes. *Gene* 2007;**404** (1):101–109.
41 Xie KY, Yang L, Chen K, Li Q. *In vitro* study of the effect of cyclic strains on the dermal fibroblast (GM3384) morphology – mapping of cell responses to strain field. *Med Eng Phys* 2012;**34** (7):826–831.
42 Ingber DE. Cellular tensegrity: defining new rules of biological design that govern the cytoskeleton. *J Cell Sci* 1993;**104**:613–627.
43 De Santis G, Lennon A, Boschetti F, *et al.* How can cells sense the elasticity of a substrate? An analysis using a cell tensegrity model. *Eur Cells Mater* 2011;**22**:202–213.
44 Dalby MJ, Gadegaard N, Oreffo RO. Harnessing nanotopography and integrin–matrix interactions to influence stem cell fate. *Nat Mater* 2014;**13** (6):558–569.
45 Ward D, Williams WA, Schapiro NE, *et al.* Focal adhesion kinase signaling controls cyclic tensile strain enhanced collagen I-induced osteogenic differentiation of human mesenchymal stem cells. *Mol Cell Biomech* 2007;**4** (4):177–178.
46 Shih YV, Tseng K, Lai H, *et al.* Matrix stiffness regulation of integrin-mediated mechanotransduction during osteogenic differentiation of human mesenchymal stem cells. *J Bone Miner Res* 2011;**26** (4):730–738.
47 Engler AJ, Sen S, Sweeney HL, Discher DE. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126** (4):677–689.
48 Gershlak JR, Black LD. Beta 1 integrin binding plays a role in the constant traction force generation in response to varying stiffness for cells grown on mature cardiac extracellular matrix. *Exp Cell Res* 2015;**330** (2):311–324.
49 Steward AJ, Wagner DR, Kelly DJ. The pericellular environment regulates cytoskeletal development and the differentiation of mesenchymal stem cells and determines their response to hydrostatic pressure. *Eur Cell Mater* 2013;**25**:167–178.
50 Müller P, Langenbach A, Kaminski A, Rychly J. Modulating the actin cytoskeleton affects mechanically induced signal transduction and differentiation in mesenchymal stem cells. *PLoS ONE* 2013;**8** (7):e71283.
51 Mathieu PS, Loboa EG. Cytoskeletal and focal adhesion influences on mesenchymal stem cell shape, mechanical properties, and differentiation down osteogenic, adipogenic, and chondrogenic pathways. *Tissue Eng Part B Rev* 2012;**18** (6):436–444.
52 Khani M, Tafazzoli-Shadpour M, Goli-Malekabadi Z, Haghighipour N. Mechanical characterization of human mesenchymal stem cells subjected to cyclic uniaxial strain and TGF-β1. *J Mech Behav Biomed Mater* 2015;**43**:18–25.
53 Swift J, Ivanovska IL, Buxboim A, *et al.* Nuclear lamin-A scales with tissue stiffness and enhances matrix-directed differentiation. *Science* 2013;**341** (6149):1240104

54 Buxboim A, Swift J, Irianto J, *et al.* Matrix elasticity regulates lamin-A, C phosphorylation and turnover with feedback to actomyosin. *Curr Biol* 2014;**24** (16):1909–1917.

55 Hoey DA, Tormey S, Ramcharan S, *et al.* Primary cilia-mediated mechanotransduction in human mesenchymal stem cells. *Stem Cells* 2012;**30** (11):2561–2570.

56 Malone AM, Anderson CT, Tummala P, *et al.* Primary cilia mediate mechanosensing in bone cells by a calcium-independent mechanism. *Proc Natl Acad Sci U S A* 2007;**104** (33):13325–13330.

57 Gardner K, Arnoczky SP, Lavagnino M. Effect of *in vitro* stress-deprivation and cyclic loading on the length of tendon cell cilia *in situ*. *J Orthop Res* 2011;**29** (4):582–587.

58 McMurray R, Wann A, Thompson C, *et al.* Surface topography regulates wnt signaling through control of primary cilia structure in mesenchymal stem cells. *Sci Rep* 2013;**3**:3545.

59 McMahon LA, Campbell VA, Prendergast PJ. Involvement of stretch-activated ion channels in strain-regulated glycosaminoglycan synthesis in mesenchymal stem cell-seeded 3D scaffolds. *J Biomech* 2008;**41** (9):2055–2059.

60 Tarbell JM, Ebong EE. The endothelial glycocalyx: a mechano-sensor and -transducer. *Sci Signal* 2008;**1** (40):pt8.

61 Coulson-Thomas VJ, Gesteira TF, Hascall V, Kao W. Umbilical cord mesenchymal stem cells suppress host rejection: the role of the glycocalyx. *J Biol Chem* 2014;**289** (34):23465–23481.

62 Hada N, Okayasu M, Ito J, *et al.* Receptor activator of NF-κB ligand-dependent expression of caveolin-1 in osteoclast precursors, and high dependency of osteoclastogenesis on exogenous lipoprotein. *Bone* 2012;**50** (1):226–236.

63 Kular J, Tickner J, Chim SM, Xu J. An overview of the regulation of bone remodelling at the cellular level. *Clin Biochem* 2012;**45** (12): 863–873.

64 Singh PP, van der Kraan, A Gabrielle J, *et al.* Membrane-bound receptor activator of NFκB ligand (RANKL) activity displayed by osteoblasts is differentially regulated by osteolytic factors. *Biochem Biophys Res Commun* 2012;**422** (1):48–53.

65 Chen Y, Shie M, Hung C Jr., *et al.* Osteoblasts subjected to tensile force induce osteoclastic differentiation of murine macrophages in a coculture system. *J Dent Sci* 2015;**10** (1):81–87.

66 Ye X, Zhang P, Xue S, *et al.* Adipose-derived stem cells alleviate osteoporosis by enhancing osteogenesis and inhibiting adipogenesis in a rabbit model. *Cytotherapy* 2014;**16** (12): 1643–1655.

67 Lu Z, Roohani-Esfahani S, Li J, Zreiqat H. Synergistic effect of nanomaterials and BMP-2 signalling in inducing osteogenic differentiation of adipose tissue-derived mesenchymal stem cells. *Nanomed Nanotechnol Biol Med* 2015;**11** (1):219–228.

68 Miao T, Rao KS, Spees JL, Oldinski RA. Osteogenic differentiation of human mesenchymal stem cells through alginate–graft–poly (ethylene glycol) microsphere-mediated intracellular growth factor delivery. *J Controlled Release* 2014;**192**:57–66.

69 Huszar JM, Payne CJ. MIR146A inhibits JMJD3 expression and osteogenic differentiation in human mesenchymal stem cells. *FEBS Lett* 2014;**588** (9):1850–1856.

70 Faia-Torres AB, Guimond-Lischer S, Rottmar M, *et al.* Differential regulation of osteogenic differentiation of stem cells on surface roughness gradients. *Biomaterials* 2014;**35** (33):9023–9032.

71 Johnstone S, Liley M, Dalby MJ, Barnett SC. Comparison of human olfactory and skeletal MSCs using osteogenic nanotopography to demonstrate bone specific bioactivity of the surfaces. *Acta Biomater* 2015;**13**:266–276.

72 Zhang Z, Wang J, Xu Y, *et al.* Menstrual blood derived mesenchymal cells ameliorate cardiac fibrosis via inhibition of endothelial to mesenchymal transition in myocardial infarction. *Int J Cardiol* 2013;**168** (2):1711–1714.

73 De Ugarte DA, Morizono K, Elbarbary A, *et al.* Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* 2003;**174** (3):101–109.

74 Lorenz K, Sicker M, Schmelzer E, *et al.* Multilineage differentiation potential of human dermal skin-derived fibroblasts. *Exp Dermatol* 2008;**17** (11):925–932.

75 Sumanasinghe RD, Osborne JA, Loboa EG. Mesenchymal stem cell-seeded collagen matrices for bone repair: effects of cyclic tensile strain, cell density, and media conditions on matrix contraction *in vitro*. *J Biomed Mater Res Part A* 2009;**88** (3):778–786.

76 Duffy CR, Zhang R, How S, *et al.* Long term mesenchymal stem cell culture on a defined synthetic substrate with enzyme free passaging. *Biomaterials* 2014;**35** (23):5998–6005.

77 Martinello T, Bronzini I, Maccatrozzo L, *et al.* Canine adipose-derived-mesenchymal stem cells do not lose stem features after a long-term cryopreservation. *Res Vet Sci* 2011;**91** (1):18–24.

78 Wall ME, Bernacki SH, Loboa EG. Effects of serial passaging on the adipogenic and osteogenic differentiation potential of adipose-derived human mesenchymal stem cells. *Tissue Eng* 2007;**13** (6): 1291–1298.

79 Wang R, Guo Y. Transient inhibition of cell proliferation does not compromise self-renewal of mouse embryonic stem cells. *Exp Cell Res* 2012;**318** (16):2094–2104.

80 Akala OO, Clarke MF. Hematopoietic stem cell self-renewal. *Curr Opin Genet Dev* 2006;**16** (5):496–501.

81 Shi Y, Sun G, Zhao C, Stewart R. Neural stem cell self-renewal. *Crit Rev Oncol* 20081; **65** (1):43–53.

82 Guo X, Wang XF. Signaling cross-talk between TGF-β/BMP and other pathways. *Cell Res* 2008;**19** (1):71–88.

83 Guo L, Zhao RC, Wu Y. The role of microRNAs in self-renewal and differentiation of mesenchymal stem cells. *Exp Hematol* 2011; **39** (6):608–616.

84 Tsai C, Su P, Huang Y, *et al.* Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell* 2012;**47** (2):169–182.

85 Lee MY, Lim HW, Lee SH, Han HJ. Smad, PI3K/Akt, and Wnt-dependent signaling pathways are involved in BMP-4-induced ESC self-renewal. *Stem Cells 2009;* **27** (8):1858–1868.

86 Qi X, Li TG, Hao J, *et al.* BMP4 supports self-renewal of embryonic stem cells by inhibiting mitogen-activated protein kinase pathways. *Proc Natl Acad Sci U S A* 2004;**101** (16):6027–6032.

87 Burdon T, Stracey C, Chambers I, *et al.* Suppression of SHP-2 and ERK signalling promotes self-renewal of mouse embryonic stem cells. *Dev Biol* 1999;**210** (1):30–43.

88 Matsuda T, Nakamura T, Nakao K, *et al.* STAT3 activation is sufficient to maintain an undifferentiated state of mouse embryonic stem cells. *EMBO J* 1999;**18** (15):4261–4269.

89 Dreesen O, Brivanlou AH. Signaling pathways in cancer and embryonic stem cells. *Stem Cell Rev* 2007;**3** (1):7–17.

90 De Vos J, Jourdan M, Tarte K, *et al.* JAK2 tyrosine kinase inhibitor tyrphostin AG490 downregulates the mitogen-activated protein kinase (MAPK) and signal transducer and activator of transcription

(STAT) pathways and induces apoptosis in myeloma cells. *Br J Haematol* 2000;**109** (4):823–828.
91 He XC, Zhang J, Tong W, *et al.* BMP signaling inhibits intestinal stem cell self-renewal through suppression of Wnt–β-catenin signaling. *Nat Genet* 2004;**36** (10):1117–1121.
92 Kolf CM, Cho E, Tuan RS. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 2007;**9** (1):204.
93 Takebe N, Harris PJ, Warren RQ, Ivy SP. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. *Nat Rev Clin Oncol* 2010;**8** (2):97–106.
94 Huang C, Tsai M, Luo J, *et al.* Suppression of androgen receptor enhances the self-renewal of mesenchymal stem cells through elevated expression of EGFR. *Biochimt Biophys Acta* 2013; **1833** (5):1222–1234.
95 Chang M, Rhee Y, Yi S, *et al.* Doxycycline enhances survival and self-renewal of human pluripotent stem cells. *Stem Cell Rep* 2014;**3** (2):353–364.
96 Raïf EM, Seedhom BB. Effect of cyclic tensile strain on proliferation of synovial cells seeded onto synthetic ligament scaffolds – an *in vitro* simulation. *Bone* 2005;**36** (3):433–443.
97 Pietramaggiori G, Liu P, Scherer SS, *et al.* Tensile forces stimulate vascular remodeling and epidermal cell proliferation in living skin. *Ann Surg* 2007;**246** (5):896–902.
98 Song G, Ju Y, Shen X, *et al.* Mechanical stretch promotes proliferation of rat bone marrow mesenchymal stem cells. *Colloids Surf B Biointerfaces* 2007;**58** (2):271–277.
99 Ghazanfari S, Tafazzoli-Shadpour M, Shokrgozar MA. Effects of cyclic stretch on proliferation of mesenchymal stem cells and their differentiation to smooth muscle cells. *Biochem Biophys Res Commun* 2009;**388** (3):601–605.
100 McBeath R, Pirone DM, Nelson CM, *et al.* Cell shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. *Dev Cell* 2004;**6** (4):483–495.
101 Wang JH, Goldschmidt-Clermont P, Wille J, Yin FC. Specificity of endothelial cell reorientation in response to cyclic mechanical stretching. *J Biomech* 2001;**34** (12):1563–1572.
102 Subramony SD, Dargis BR, Castillo M, *et al.* The guidance of stem cell differentiation by substrate alignment and mechanical stimulation. *Biomaterials* 2013;**34** (8):1942–1953.
103 Foolen J, Deshpande VS, Kanters FMW, Baaijens FPT. The influence of matrix integrity on stress-fiber remodeling in 3D. *Biomaterials* 2012;**33** (30):7508–7518.
104 Han WM, Heo S, Driscoll TP, *et al.* Macro-to microscale strain transfer in fibrous tissues is heterogeneous and tissue-specific. *Biophys J* 2013;**105** (3):807–817.
105 Li Y, Huang G, Zhang X, *et al.* Engineering cell alignment *in vitro*. *Biotechnol Adv* 2014;**32** (2):347–365.
106 Sabuncuoglu B, Acar M, Silberschmidt VV. Finite element modelling of fibrous networks: analysis of strain distribution in fibres under tensile load. *Comput Mater Sci* 2013;**79**:143–158.
107 Xu F, Beyazoglu T, Hefner E, *et al.* Automated and adaptable quantification of cellular alignment from microscopic images for tissue engineering applications. *Tissue Eng Part C Methods* 2011;17 (6):641–649.
108 Sumanasinghe RD. Functional bone tissue engineering using human mesenchymal stem cells and polymeric scaffolds. PhD dissertation, ProQuest; 2006.
109 Kearney E, Farrell E, Prendergast P, Campbell V. Tensile strain as a regulator of mesenchymal stem cell osteogenesis. *Ann Biomed Eng* 2010;**38** (5):1767–1779.
110 Cope JB, Yamashita J, Dechow PC, Harper RP. Force level and strain patterns during bilateral mandibular osteodistraction. *J Oral Maxillofac Surg* 2000;**58** (2):171–178.
111 Cillo JE Jr., Gassner R, Koepsel RR, Buckley MJ. Growth factor and cytokine gene expression in mechanically strained human osteoblast-like cells: implications for distraction osteogenesis. *Oral Surg Oral Med Oral Pathol Oral Radiol Endodontol* 2000;**90** (2):147–154.
112 Banka S, Mukudai Y, Yoshihama Y, *et al.* A combination of chemical and mechanical stimuli enhances not only osteo- but also chondro-differentiation in adipose-derived stem cells. *J Oral Biosci* 2012;**54** (4):188–195.
113 Engelmayr GC Jr., Sales VL, Mayer JE Jr., Sacks MS. Cyclic flexure and laminar flow synergistically accelerate mesenchymal stem cell-mediated engineered tissue formation: implications for engineered heart valve tissues. *Biomaterials* 2006;**27** (36):6083–6095.
114 Doroski DM, Levenston ME, Temenoff JS. Cyclic tensile culture promotes fibroblastic differentiation of marrow stromal cells encapsulated in poly(ethylene glycol)-based hydrogels. *Tissue Eng Part A* 2010;**16** (11):3457–3466.
115 Colazzo F, Sarathchandra P, Smolenski RT, *et al.* Extracellular matrix production by adipose-derived stem cells: implications for heart valve tissue engineering. *Biomaterials* 2011;**32** (1):119–127.
116 Kim J, Choi YS, Lim S, *et al.* Comparative analysis of the secretory proteome of human adipose stromal vascular fraction cells during adipogenesis. *Proteomics* 2010;**10** (3):394–405.
117 Choi S, Kim J, Yea K, *et al.* Targeted label-free quantitative analysis of secretory proteins from adipocytes in response to oxidative stress. *Anal Biochem* 2010;**401** (2):196–202.
118 Frazier TP, Gimble JM, Kheterpal I, Rowan BG. Impact of low oxygen on the secretome of human adipose-derived stromal/stem cell primary cultures. *Biochimie* 2013;**95** (12):2286–2296.
119 Giusta M, Andrade H, Santos A, *et al.* Proteomic analysis of human mesenchymal stromal cells derived from adipose tissue undergoing osteoblast differentiation. *Cytotherapy* 2010;**12** (4):478–490.
120 Ando Y, Matsubara K, Ishikawa J, *et al.* Stem cell-conditioned medium accelerates distraction osteogenesis through multiple regenerative mechanisms. *Bone* 2014;**61**:82–90.
121 Adamowicz J, Pokrywczyńska M, Drewa T. Conditioned medium derived from mesenchymal stem cells culture as a intravesical therapy for cystitis interstitials. *Med Hypotheses* 2014;**82** (6): 670–673.
122 Lavoie JR, Rosu-Myles M. Uncovering the secretes of mesenchymal stem cells. *Biochimie* 2013;**95** (12):2212–2221.
123 Stegen S, van Gastel N, Carmeliet G. Bringing new life to damaged bone: the importance of angiogenesis in bone repair and regeneration. *Bone* 2015;**70**:19–27.
124 Seebach E, Freischmidt H, Holschbach J, *et al.* Mesenchymal stroma cells trigger early attraction of M1 macrophages and endothelial cells into fibrin hydrogels, stimulating long bone healing without long-term engraftment. *Acta Biomater* 2014; **10** (11):4730–4741.
125 Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3** (4):e1886.

126 Walter M, Wright KT, Fuller H, *et al.* Mesenchymal stem cell-conditioned medium accelerates skin wound healing: An *in vitro* study of fibroblast and keratinocyte scratch assays. *Exp Cell Res* 2010;**316** (7):1271–1281.

127 Bozorgmehr M, Moazzeni SM, Salehnia M, *et al.* Menstrual blood-derived stromal stem cells inhibit optimal generation and maturation of human monocyte-derived dendritic cells. *Immunol Lett* 2014;**162** (2):239–246.

CHAPTER 18

# The evolving concept of mesenchymal stromal cells in regenerative medicine: from cell differentiation to secretome

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## 18.1 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs), derived from mesenchymal stem cells, have been suggested as possible therapeutic tools for regenerative medicine [1–3]. Indeed, when compared with other sources, they do not have the ethical and moral questions raised by embryonic stem cells or the technical issues regarding the isolation and further *in vitro* expansion of, for instance, other sources, such as neural stem cells [2,4]. This great potential has been associated with their widespread availability throughout the human body, along with the fact that, when isolated, they display great proliferative potential with minimal senescence through multiple passages [5,6]. In 2006, the International Society for Cell Therapy introduced the minimal criteria for the identification of MSCs, defining them as a plastic-adherent multipotent population, with the ability to differentiate *in vitro* down the osteogenic, chondrogenic, and adipogenic lineages, displaying CD105, CD90, and CD73 surface markers, while being negative for typical hematopoietic markers such as CD45 and CD34 [7]. Following the pioneering work of Friedenstein *et al.* [8], several reports have confirmed that MSCs are not only present within the bone marrow, but also in other sources in the body, including adipose tissue [9,10], dental pulp [11,12], placenta [13,14], umbilical cord blood [15] and Wharton's jelly [16,17], and brain [18]. Wharton's jelly is a gelatinous substance within the umbilical cord largely composed of the mucopolysaccharides hyaluronic acid, and chondroitin sulfate. It also contains some fibroblasts and macrophages. Thomas Wharton was an English physician and anatomist (1614–1673) who first described it in his publication *Adenographia*, or "The Description of the Glands of the Entire Body," first published in 1656.

Thus, there has been an increasing interest in MSCs as candidates for new therapeutic strategies [19–22]. In fact, over the last decade, there has been a substantial effort to assess the impact of MSCs into different types of disease [23–28]. Studies have demonstrated that these cells are able to induce a significant recovery in some diseases, such as graft-versus-host-disease (GVHD) following allogeneic hematopoietic stem cell transplantation (HSCT) [27], acute lung injury [26], peripheral nerve injury [29] and neurodegenerative disorders such as stroke [30,31], demyelination [32,33], Parkinson's disease [34,35] and spinal cord injury (SCI) [36,37]. Despite these observations, the main mechanisms mediating these effects of MSCs are still a matter of intense debate [38]. Parkinson's disease is named after the English doctor James Parkinson, who published the first

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detailed description of the disease in *An Essay on the Shaking Palsy* in 1817.

Initially it was believed that the therapeutic potential of these cells relied on their multilineage differentiation ability [39]. Indeed, most of the literature of the 1990s and early 21st century focused on the differentiation of these cells down mesodermal lineages, often within three-dimensional (3D) matrices as scaffolds. Around the same time it was also suggested that MSCs had an even greater differentiation potential than was originally predicted, as several reports indicated that these cells could be differentiated beyond the mesodermal lineages [7]. However, the real nature of the differentiated cells in the observed regenerative processes was never fully revealed [40,41]. In 2005, Gnechhi *et al.* [42] put forward a new concept that would change the paradigm of how MSCs could be used in regenerative medicine, by showing that their therapeutic potential was mostly related to the growth factors that they secreted into the extracellular milieu, rather than to their differentiation potential [42]. Since then different reports have revealed the role of the MSC secretome in controlling key processes such as cell proliferation, survival, differentiation, and extracellular matrix deposition, among others [38,43–46]. Recently, it has even been proposed that the secretome alone, with no need for cell transplantation, could be used for therapeutic purposes [47]. In fact, Teixeira *et al.* [47] have recently shown that animals injected with the secretome of MSCs isolated from the Wharton's jelly component of the umbilical cord perivascular cells into the hippocampal dentate girus displayed similar levels of proliferation, neuronal/glial survival, and differentiation, when compared with those transplanted with MSCs.

With this in mind, we will discuss in this chapter the current understanding of the MSC secretome and its possible applications to regenerative medicine. We will focus particularly on the secretome composition and characterization, as well as its application in GVHD and disorders of the nervous system (Figure 18.1).

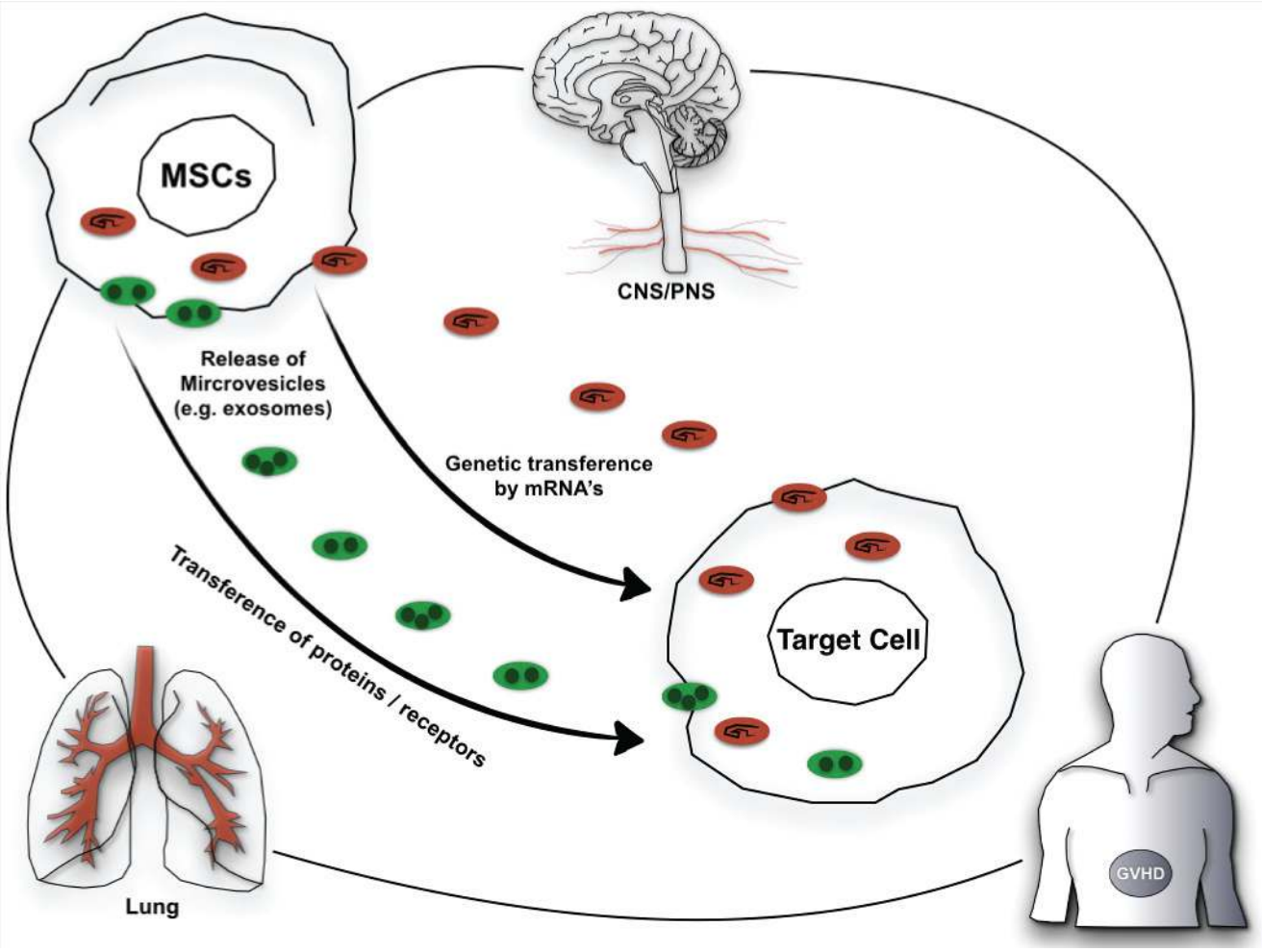



**Figure 18.1** The interactions between MSCs and their target tissues.

## 18.2 The mesenchymal stromal cell secretome

### 18.2.1 Concept

Over the last several years it has become evident that some therapeutic effects mediated by MSCs are mainly associated with the secretion of bioactive molecules and vesicles, which constitute their secretome [48]. The definition of secretome has not yet been completely defined; however, one definition is that it comprises the proteins or vesicles (microvesicles or exosomes) that are released by a cell, tissue, or organism that are subsequently crucial for the regulation of different cell processes. In this sense, studies have suggested that, in response to injury, MSCs have the capacity to migrate to the site of damage, leading to the promotion of regenerative processes through the secretion of trophic molecules such as growth factors, cytokines, and antioxidants [49,50]. Indeed, despite the capacity of cell–cell interaction of MSCs with their surrounding microenvironment, their secretome seems to be the main effector of their immunomodulatory and regenerative capacity at the site of the lesion [51,52]. As a consequence of this, Caplan and Dennis [53] have designated MSCs as trophic mediators of regenerative processes. In addition to this, it has also been demonstrated that MSCs from different sources can secrete important trophic molecules such as brain-derived neurotrophic factor (BDNF), β-nerve growth factor (β-NGF), insulin-like growth factor-1 (IGF-1), hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), transforming growth factor-β, glial cell line-derived neurotrophic factor (GDNF), fibroblast growth factor-2 (FGF-2), stem cell factor (SCF), granulocyte colony-stimulating factor, and stroma-derived factor-1 (SDF-1) (also known as CXCL12) both *in vitro* and *in vivo* in different disease contexts [3,48,49,53–55]. In fact, the latter have been considered important mediators of MSC action in a variety of cell processes, including immunomodulatory capacity, inhibition of apoptosis, promotion of angiogenesis, and stimulation of adjacent cells [53]. In addition to these soluble paracrine factors, recent findings have shown that an important component of their secretome is constituted in vesicular bodies, known as microvesicles or exosomes [56,57]. Indeed, robust data from the literature indicate that MSCs are able to secrete large amounts of these vesicles either constitutively or after signal activation [56]. These vesicles secreted by MSCs have been considered as a vehicle of paracrine or endocrine signaling, able to transport defined signaling molecules to distant target cells or tissues [58–60].

The biochemical composition and the complex biogenesis of these vesicles, as well as their physiological roles, have not been fully elucidated [57,61]. Their potential as mediators of cell-to-cell communication has been demonstrated. They have demonstrated features such as the ability to transfer proteins and functional genetic material such as microRNAs to other cells [62,63]. The release of exosomes has received much attention. The secretion of these nanovesicles was first described by Harding *et al.* during the maturation of sheep reticulocytes, who considered them necessary to remove unneeded proteins from the cells [64]. Typically, exosomes are formed by inward budding of late endosomes, having a diameter of 40–10 nm, with a density of 1.13–1.19 g/mL in a sucrose solution [65]. A unique function associated with exosomes is their capacity to interact with targeted cells, which putatively enable cell-to-cell communications in physiological processes such as antigen presentation, genetic exchange, immune responses, and angiogenesis [65]. MSCs have recently been considered an efficient mass producer of exosomes [66]. Indeed, it has been suggested that MSCs can produce higher amounts of exosomes than other types of cell or cell lines, including myoblasts, the human acute monocytic leukemia cell line THP-1, and the human embryonic kidney cell line [66]. Characteristically, MSC-derived exosomes express their common cell surface markers such as CD9, CD63, and CD81 as well as adhesion molecules such as CD29, CD44, and CD73, which are also expressed on the cell surface membrane of MSCs [65].

The amount of these vesicles in the secretome of MSCs seems to be highly variable from batch to batch. Lai *et al.* [63] demonstrated by liquid chromatography–tandem mass spectrometry that MSC-derived exosomes obtained from three different MSC batches contained different proteins, with only a few common to each batches. The inclusion of all these proteins suggests that MSC-derived exosomes have the potential to drive a number of different cellular processes [65,67–70].

MSC-derived exosomes have demonstrated promising results in a wide range of diseases [26,27,67,71–77]. Thus, despite knowledge gaps regarding the exosomal/vesicular fraction of the MSC secretome, studies have already demonstrated that MSC-derived exosomes, when compared with whole cells, are more stable and present a lower risk of aneuploidy and immune rejection after *in vivo* allogenic administration, thus themselves representing a potential therapeutic tool for the treatment of a number of different diseases [65].

### 18.2.2 Characterization techniques

Although important progress has been made on the characterization of the MSC secretome in the last 10 years, only the "tip of the iceberg" has been uncovered. It is therefore important to further characterize it, using state-of-the-art techniques to provide a broad overview of its composition. By doing this, it may possible to understand fully the mechanisms by which the MSC secretome mediates its effects.

Proteomic-based techniques have been the most widely used methodology for this purpose [78,79]. Proteomics has been defined as a discipline focused on the large-scale analysis of proteins and their physiological functions [79]. Typically, such analysis is performed on the conditioned medium (CM) of cultured MSCs. To date, different proteomic techniques such as protein separation techniques (two-dimensional gel electrophoresis (2-DE) or liquid chromatography), mass spectrometry (MS), and antibody-based methods (enzyme-linked immunosorbent assay (ELISA) or multiplex assay) have been used to

obtain a broad characterization of the MSC secretome. Two-dimensional gel electrophoresis or liquid chromatography is necessary for proteome analysis of most mammalian cell secretomes (including that of MSCs) since mammalian cell secretomes comprise a large number of different proteins with broad dynamic ranges [80].

#### 18.2.2.1 Two-dimensional gel electrophoresis, sodium dodecyl sulfate polyacrylamide gel electrophoresis, and mass spectrometry

Using 2-DE, the proteins in the MSC secretome are separated according to their isoelectric points, followed by use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), which allows separation of different proteins based on their molecular weight [81,82]. In addition to this first step, the use of Coomassie blue, silver nitrate, or fluorescent dyes are subsequently used to identify protein spots in the gels [79]. After this, spots of interest are removed, alkylated, and enzymatically digested into peptides for subsequent protein identification by MS [83]. This methodology is considered the most powerful methodology in the separation of translational modifications and isoforms of specific proteins [84]. An example of this is the recent report by Fraga *et al.* [46], in which 2-DE and MS were used to characterize the secretome of MSCs isolated from the Wharton's jelly of the umbilical cord. Its limitations are mainly related to the solubilization and analysis of highly hydrophobic proteins or proteins with high molecular weights.

#### 18.2.2.2 Liquid chromatography

Proteins or peptides can also be isolated by liquid chromatography [79]. This technique may overcome some of the limitations of 2-DE [85,86]. Liquid chromatography is the most common technique used in the analysis of the MSC secretome, by chromatographically separating proteins digested into peptides [85]. Reversed-phase liquid chromatography (RPLC) is often used, providing a reliable peptide separation of CM samples [87,88]. To increase the accuracy of the separation of the peptides, a combination of two RPLC runs using different pH values during the mobile phases is performed [85,87,88]. The final product of this process is the achievement of a dissected panel of peptides, which can be directly analyzed in a mass spectrometer allowing the identification of proteins within the CM samples [86].

#### 18.2.2.3 Mass spectrometry

MS is a powerful technique for the identification of proteins in serum-free CM samples through the exact determination of molecular mass of the peptides [89,90]. Protein identity is assigned using a protein database, comparing the CM samples being analyzed with the mass data obtained [91,92]. There have been recent improvements in MS, including stable isotope labeling by amino acids in cell culture, isotope-coded affinity tagging, isobaric tagging for relative and absolute quantitation, selected reaction monitoring, and spectral counting or ion intensity measurement, allowing the relative quantification of proteins in the CM samples [90,93–97]. The performance of these different techniques allows a direct comparison of proteins among samples (e.g., CM from different MSC sources), thus providing more reliable and comprehensive information about protein expression of MSCs [79].

#### 18.2.2.4 Enzyme-linked immunosorbent assay

Finally, in addition to all the techniques described so far, the use of immunological assays such as ELISA and multiplex assays have also been described as promising tools in the proteomic field [79]. The advantages of these techniques is based on the use of specific antibodies, allowing higher sensitivity levels in the detection of antigens, and thus enabling the identification of proteins present in low concentrations, such as cytokines [79].

ELISA (or Western blot) can easily be used for the detection of a single analyte (i.e., in this case a protein of interest), and can provide accurate quantification of proteins when compared with calibration standards.

#### 18.2.2.5 Multiplex assay

A multiplex assay is a type of assay that simultaneously measures multiple analytes (dozens or more) in a single run/cycle of the assay. It is distinguished from procedures that measure one analyte at a time. Multiplex assays within a given application area or class of technology can be further stratified based on how many analytes can be measured per assay, where "multiplex" refers to those with the highest number of analyte measurements per assay (up to millions), and "low-plex" or "mid-plex" refers to procedures that process fewer (tens to thousands), though there are no formal guidelines for calling a procedure multi-, mid-, or low-plex based on number of analytes measured. The use of multiplex techniques in proteomics has been developed and the technique is a powerful tool capable of detecting and quantifying a wide panel of analytes across multiple and different samples [79,98]. This is based on the comparison of the fluorescence intensity of the samples with that of the calibration standards, allowing a broad and reliable dynamic range of detection [79].

## 18.3 The mesenchymal stromal cell secretome in transplantation and regenerative medicine

### 18.3.1 Graft-versus-host-disease

Allogeneic HSCT is most commonly used to treat hematological malignancy for which no other potentially curative procedure is available. GVHD remains a major cause of morbidity and mortality in allogeneic HSCT recipients, even when the donor and recipient have a high level of human leukocyte antigen compatibility. It commonly affects the skin, gastrointestinal tract and liver, and the acute form of the disease, by definition, presents within 100 days of the transplant. The pathophysiology of acute GVHD is characterized by three well-established stages. The first stage, commonly known as "the cytokine storm," is a

result of the high-dose chemotherapy or chemoradiation conditioning regimen given to the recipient before the transplant in order to eradicate the underlying disease, as well as eradication of the recipient's immune system, so that the recipient will not reject the donor transplant. The second stage involves cellular activation and is characterized by activation of donor T cells by recipient cytokines and antigen-presenting cells not completely eradicated by the pretransplant conditioning regimen. In the third stage, known as the "effector" stage, T cells start to damage the recipient's tissues [99,100]. As the etiology of GVHD involves an allogeneic cytotoxic reaction by donor T lymphocytes, the cornerstone treatment is immunosuppression, with the purpose of inducing donor/recipient tolerance without eliminating the therapeutically valuable graft-versus-malignancy effect [101]. Prophylactic immune suppression is routinely used to minimize the severity of GVHD and usually involves the use of methotrexate and a calcineurin inhibitor such as cyclosporine or tacrolimus. If GVHD develops in spite of prophylactic immune suppression, corticosteroids are the first-line therapy of choice [102]. A recent study confirmed that early response to corticosteroids is associated with increased survival [100]. However, only 60–70% of patients with GVHD respond to standard corticosteroid therapy. Moreover, patients with severe or steroid-refractory GVHD have few therapeutic alternatives, no established treatment protocol, and a 2-year survival as low as 10% [103].

It is in this context that MSCs have been used as treatment for corticosteroid-refractory GVHD. The rationale behind this is the known immunomodulatory repertoire of their secretome. For instance, Ringden and co-workers [104,105] reported that approximately half of patients with life-threatening acute GVHD, who were not responding to conventional immunosuppressive therapy, responded to treatment with MSCs. Additionally, it was also observed that family member MSCs or MSCs from unrelated donors were equally effective, and that children tended to have a better response than adults [104]. Furthermore, tissue toxicity, such as hemorrhagic cystitis, pneumomediastinum, and colon perforation with peritonitis, could also be reversed by their administration [104]. Similarly, Le Blanc and colleagues reported that the administration of haploidentical MSC infusions in a 9-year-old boy with severe (grade IV) acute GVHD of the gastrointestinal tract and liver also resulted in a successful outcome [106]. Kurtzberg *et al.* also reported a successful approach in children with GVHD: a 64% response rate was obtained in 59 children 28 days after infusion of MSCs from unrelated donors [107].

### 18.3.2 The central nervous system

Several studies have demonstrated, that, beyond being able to survive in the tissues of the central nervous system (CNS), MSCs are also able to secrete a wide panel of neuroregulatory molecules that leads to the enhancement of neuronal cell survival and neuritogenesis both *in vitro* and *in vivo* [45,108–110]. In addition to this, the modulation of inflammatory brain reactions and potentiation of axonal regeneration/remyelination have been associated with the use of MSCs in preclinical models of disease [33,111]. In models of Parkinson's disease and SCI, studies have demonstrated that MSCs can secrete important trophic factors, including BDNF, FGF-2, GDNF, and IGF-1. This may have been the reason for the increase in neuronal survival and regeneration noted as well as the improvement of animal performance after the administration of MSCs [111–118].

#### 18.3.2.1 Spinal cord injury

SCI is characterized by long-term functional deficits in ascending and descending motor and sensory neuronal pathways as a result of injury. In most cases it leads to a complex cascade of events that result in loss of neurons and glial cells, inflammation, demyelination, and pain.

MSC treatment has shown potential promise for SCI [36,119]. Despite the complexity of SCI lesions, transplantation with MSCs derived from bone marrow has shown that these cells were able to promote remyelination, axonal sparing, and functional recovery at different stages of SCI [120–122]. Besides the fact that MSCs are immunosuppressive, studies have shown that they can modify the SCI milieu directly through the release of trophic factors such as BDNF, NGF, and VEGF, thus promoting axonal regeneration, neurite outgrowth, and glial scar reduction [110,123]. Lu *et al.* [124] showed that bone marrow-derived MSCs secreted NGF, neurotrophin-3 (NT-3), and high levels of BDNF, contributing to host axonal growth and enhancing the growth of the host's serotonergic, coerulospinal, and dorsal column sensory axons after SCI. Similar findings were also reported by Neuhuber *et al.* [125], who demonstrated that the CM of bone marrow-derived MSCs was able to promote axon growth and functional recovery due to the presence of BDNF, VEGF, interleukin (IL)-6, monocyte chemoattractant protein-1, SCF, and SDF-1α. Recently, Gu *et al.* [111] and Park *et al.* [126] showed that these MSCs were able to secrete neurotrophic factors such as HGF, VEGF, BDNF, and GDNF, suggesting that this secretory activity was possibly the mechanism of the promotion of axonal regeneration of spinal neurons both *in vitro* and *in vivo*.

Adipose-tissue-derived stem cells (which contain MSCs) were shown to be similar to Schwann cells, secreting neurotrophic factors such as BDNF and improving remyelination [115]. Moreover, undifferentiated adipose-tissue-derived stem cells may be another promising cellular candidate for axonal regeneration that has been associated with their paracrine action [36]. Two studies have examined the use of Wharton's jelly MSCs in SCI: their transplantation into the site of SCI was able to potentiate repair and recovery due to the release of trophic factors such as NT-3, VEGF, basic FGF (bFGF), and BDNF [116,127].

More recently, Cantinieaux *et al.* [128] used an original approach: instead of transplanting MSCs, he and his colleagues administered only their secretome, in the form of CM, to animals with SCI. This CM improved motor recovery, thus indicating that the use of MSC secretome as a cell-free therapeutic agent is a possibility [128].

Owing to these results, some early clinical studies have already been reported. Saito *et al.* [129] demonstrated that the transplantation of autologous bone marrow-derived MSCs by lumbar puncture into the spinal fluid appeared to be safe and with some improvement in motor function. Similar results were also obtained by Karamouzian *et al.* [130] in subacute SCI: after the MSC transplantation the authors observed that 45.5% of patients showed improvements in neurological function [130].

#### 18.3.2.2 Parkinson's disease

Parkinson's disease is the most common motor-related disorder of the CNS in middle or late life and affects millions of people worldwide [131]. The disease is named after the English doctor James Parkinson, who published the first detailed description of the disease in *An Essay on the Shaking Palsy* in 1817 (Figure 18.2).

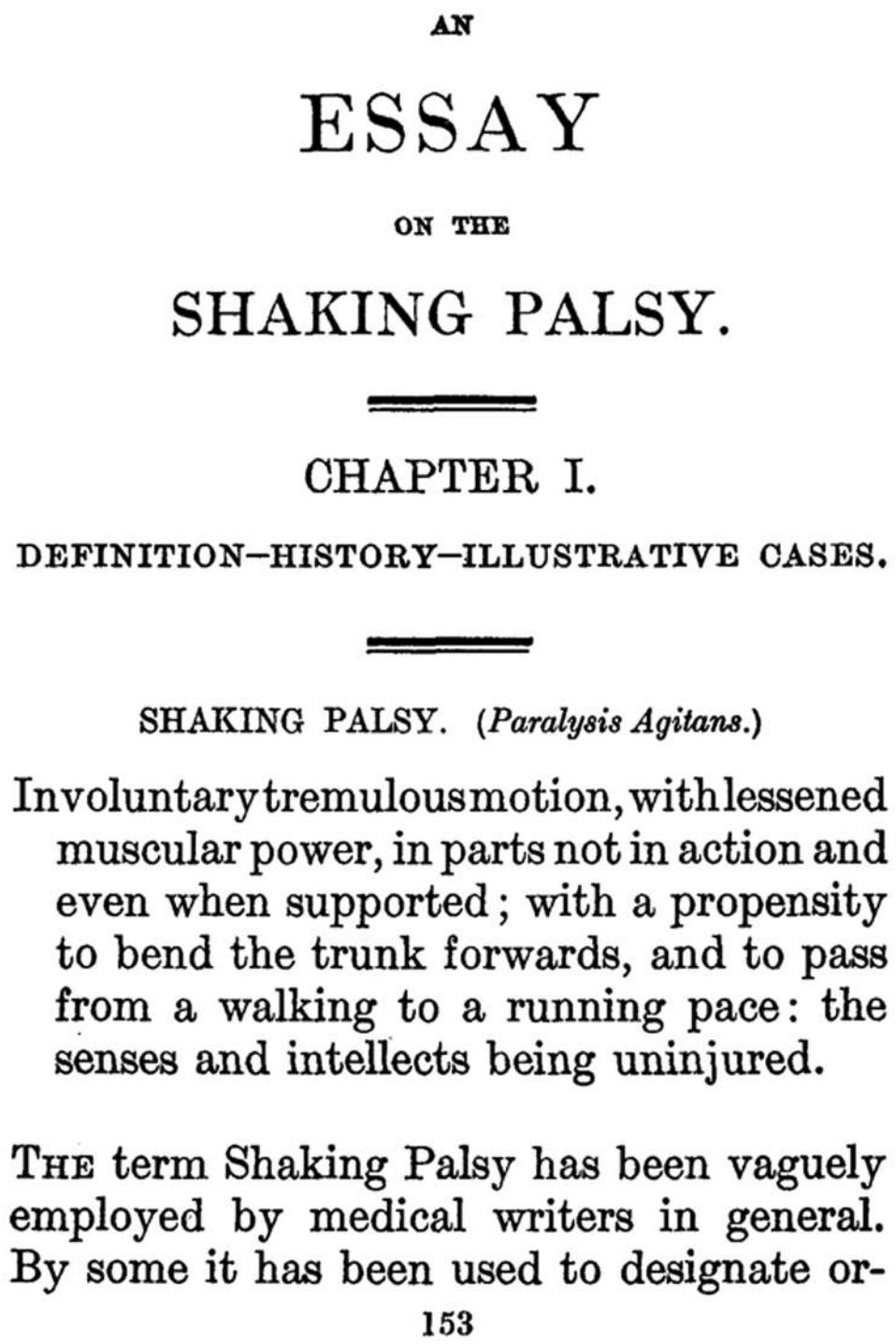
Parkinson's disease results in loss of dopaminergic innervation to the striatum (and other basal ganglia) and a cascade of subsequent consequences. The striatum, also known as the neostriatum or striate nucleus, is a subcortical part of the forebrain. It receives input from the cerebral cortex and is the primary input to the basal ganglia system. In all primates, the striatum is divided by a white matter tract called the internal capsule into two sectors called the caudate nucleus and the putamen. The term corpus striatum is occasionally used to refer to the striatum combined with the pallidum, which is a collective term for the dorsal pallidum (globus pallidus) and the ventral pallidum. The lenticular nucleus refers to the putamen together with the globus pallidus. Functionally, the striatum helps coordinate body movements with motivation. Coordinating motivation and body movement can be as simple as controlling fine-motor functions or as complex as inhibiting behavior depending upon social interactions.

Parkinson's disease is a slowly progressive neurodegenerative disease that is characterized by the loss of dopaminergic neurons in several dopaminergic networks, most intensively in the ventral tier of the substantia nigra pars compacta within the mesostriatal/nigrostriatal pathway [38,131–133]. The depletion of substantia nigra neurons leads to the loss of dopaminergic innervations and consequently to striatal dopamine deficiency, which is responsible for the major sensory and motor system symptoms [134]. The motor symptoms of Parkinson's disease result from death of the dopamine-generating cells in the substantia nigra, a region of the midbrain; the cause of this cell death is unknown. Early in the course of the disease, the most obvious symptoms are movement related; these include shaking, rigidity, slowness of movement, and difficulty with walking and gait. Later, thinking and behavioral problems may arise, with dementia commonly occurring in the advanced stages of the disease, whereas depression is the most common psychiatric symptom. Other symptoms include sensory, sleep, and emotional problems. Parkinson's disease is more common in older people, with most cases occurring after the age of 50 (Figure 18.3).

A considerable body of evidence has revealed the potential of MSCs to promote protection and/or recovery of dopaminergic neurons against neurotoxin-induced nigrostriatal degeneration. Several studies in animal models of Parkinson's disease have demonstrated that bone marrow-derived MSC secretome protects and/or helps dopaminergic neurons regenerate *in vitro* and *in vivo* models through the secretion of growth factors and cytokines [34,113,135–142]. For instance, Shintani *et al.* demonstrated that CM from bone marrow-derived MSCs was able to promote survival of tyrosine hydroxylase-positive dopaminergic neurons in rat primary cultures of ventral mesencephalic cells [136]. Moreover, intrastriatal transplantation of fetal mesencephalic cells, treated with CM from human bone marrow-derived MSCs during donor cell preparation and implantation, induced survival of dopaminergic transplanted cells and promoted functional recovery in a 6-hydroxydopamine (6-OHDA) neurotoxin rat model of Parkinson's disease [136]. The observed protection of dopaminergic neurons was attributed to the secretion of BDNF, GDNF, and bFGF by the MSCs, all of which had previously been shown to stimulate survival of dopaminergic neurons [136,143–147]. Similarly, Sadan *et al.* showed that human bone marrow-derived MSCs cultured in the presence of growth factors not only significantly increased the viability of the human-derived SH-SY5Y neuroblastoma cell line exposed to 6-OHDA, but also that MSCs transplanted into the striatum of a 6-OHDA rat model of Parkinson's disease migrated to the lesion site, increased the numbers of tyrosine hydroxylase-positive cells, and increased dopamine levels [137]. These neuroprotective and neuroregenerative effects were accompanied by an improvement in animals' behavioral impairments and were correlated with MSC secretion of BDNF and GDNF. Similar findings were published by Blandini *et al.* using the same animal model [139].

In contrast, Wang *et al.* associated SDF-1α expression by rat bone marrow-derived MSCs with protection of dopaminergic neurons against the 6-OHDA neurotoxin, both *in vitro* and *in vivo*, through antiapoptotic-based mechanisms [140]. Cova *et al.* demonstrated that human bone marrow-derived MSCs transplanted in the striatum of a 6-OHDA rodent model of Parkinson's disease were able to survive and interact with tissue surrounding the lesion site, thus enhancing the survival of dopaminergic terminals and neurogenesis in the subventricular zone (SVZ) in a sustained manner [34]. The SVZ is a paired brain structure situated throughout the lateral walls of the lateral ventricles. It is composed of four distinct layers of variable thickness and cell density, as well as cellular composition. Along with the dentate gyrus of the hippocampus, the SVZ is one of two places where neurogenesis has been found to occur in the adult mammalian brain.

Finally, the secretion of BDNF *in vivo* by bone marrow-derived MSCs was correlated with the activation of endogenous stromal cells, as described by Cova *et al.* [34]. In addition to the capability of MSCs to induce survival of dopaminergic



AN

ESSAY

ON THE

SHAKING PALSY.

CHAPTER I.

DEFINITION—HISTORY—ILLUSTRATIVE CASES.

SHAKING PALSY. (*Paralysis Agitans.*)

Involuntary tremulous motion, with lessened muscular power, in parts not in action and even when supported; with a propensity to bend the trunk forwards, and to pass from a walking to a running pace: the senses and intellects being uninjured.

THE term Shaking Palsy has been vaguely employed by medical writers in general. By some it has been used to designate or-

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**Figure 18.2** First page of the landmark work by James Parkinson (1755–1824) *An Essay on the Shaking Palsy*, providing in 1817 the first description of the disease that would bear his name. *Source:* typeset and printed in 1817 by Whittingham & Rowland of London. The image reproduced here is from en.wikipedia.org. The image is in the public domain.

neurons, its effects have also been related with their immunomodulatory properties. In this context, Danielyan *et al.* [141] showed that rat bone marrow-derived MSCs delivered intranasally into 6-OHDA-induced hemi-Parkinsonian rats migrated towards the substantia nigra and the striatum and reduced the overall expression of pro-inflammatory cytokines, such as IL-1β, IL-2, IL-12, tumor necrosis factor-α, and interferon-8. Moreover, their presence also reversed the loss of nigral dopaminergic neurons and striatal fibers [141].



**Figure 18.3** Front and side views of a man portrayed to be suffering from Parkinson's disease. These are woodcut reproductions (of two collotypes from Paul de Saint-Leger's 1879 doctoral thesis, "Paralysie agitante (maladie de Parkinson); étude clinique") published in Gowers, William Richard (1886). "Fig 145 – Paralysis agitans. (After St. Leger.)." This image, reproduced here from en.Wikimedia.org, is in the public domain. The two sketches (one from the front and one from the right side) are of a man, with an expressionless face. He is stooped forward and is presumably having difficulty walking.

## 18.4 The peripheral nervous system

Following injury the axons of the peripheral nervous system (PNS) and the CNS respond differently. While CNS regeneration is limited, severed PNS axons are able to re-extend and re-innervate their targets. However, a complete recovery of nerve function is unusual and clinical outcomes remain unsatisfactory, especially for large lesions. Injuries to the PNS are one of the major sources of disability in the USA [148], impairing the ability to move muscle and/or resulting in painful neuropathies. The standard clinical procedure after a nerve trauma is the simple suture, without generating tension, of the nerve ends. However, the absence of tension is impossible when suturing large gaps. In these cases, a nerve autograft is typically performed [149]. Although the use of autografts is currently the best clinical treatment available, this procedure has many drawbacks, including the need of a second surgery, the loss of donor site function, and limited availability. The presence of inhibitory molecules in autografts may reduce their efficacy [149,150]. Therefore, the pursuit for alternative therapies that can successfully repair peripheral nerves is important.

The trophic action and application of MSCs for PNS injuries has been demonstrated [115,151–156]. For instance, Pan *et al.* [157] demonstrated that, after administration at the site of nerve injury, MSCs were able to secrete higher levels of BDNF, GDNF, ciliary neurotrophic factor, NGF, and NT-3, leading to the reduction of fibrosis in the lesion site and to the promotion of nerve regeneration. Similar observations were also observed by Lopatina *et al.* [115], who demonstrated with MSCs derived from adipose tissue that their transplantation was able to accelerate nerve regeneration through the active secretion of BDNF, enabling a better nerve sprout growth *in vivo*. Similar findings were also reported by Duan *et al.* [151], after transplanting MSCs into an acute peripheral nerve traction model in rabbits.

Taking a different approach, Hu *et al.* [152] used chemically extracted acellular allogenic nerve segments implanted with autologous MSCs to repair a 40 mm defect in a rhesus monkey ulnar nerve injury model. Results revealed that severely damaged ulnar nerves were structurally and functionally repaired within 6 months following placement of the MSC-seeded allografts in all animals studied (six of six) [152].

Recently, mesenchymal-like stromal cells isolated from adipose tissue were used to target PNS injury [158,159]. It was found that implantation of these cells improved recovery of exercise behavior and increased wet weight ratio of the anterior tibial

muscle [158]. Moreover, electrophysiological recording found that the percentage of activated fibers was higher in animals given the cells. Histologically, the number of nerve fibers, axonal diameter, and myelin thickness were significantly higher in the groups receiving cells [158]. Additionally, they demonstrated that the progression of the regenerative process after cell implantation was accompanied by elevated expression of neurotrophic factors during both early and later phases [158].

Finally, Wrobel *et al.* [160] proposed the use of MSCs in a co-culture with Schwann cells for peripheral nerve repair. Higher neurite outgrowth from dissociated sensory neurons was noted.

## 18.5 Future perspectives

The use of MSCs has been suggested as a possible therapeutic tool for regenerative medicine, representing a promising cell source for the creation of new cell-based therapies [3,21,161,162] (Figure 18.4). Promising results have been described in this chapter for disorders of the CNS, the PNS, and in GVHD. Trophic activity of MSCs has also been described in kidney injury, bone disease, heart disease, diabetes mellitus, and cancer [19,163,164]. It is proposed that the effects of bioactive molecules secreted by MSCs can have a direct effect by intracellular signaling or an indirect effect through the activation of adjacent cells, stimulating the secretion of functionally active agents by them [165]. Studies have suggested that the use of the MSC secretome may have an application in a number of diseases, including neurodegenerative diseases [130,166]. Efforts should be targeted to fully characterizing the MSC secretome, analyzing their two fractions (protein and vesicular) in order to determine which fraction will be more useful as a therapeutic strategy in the future.

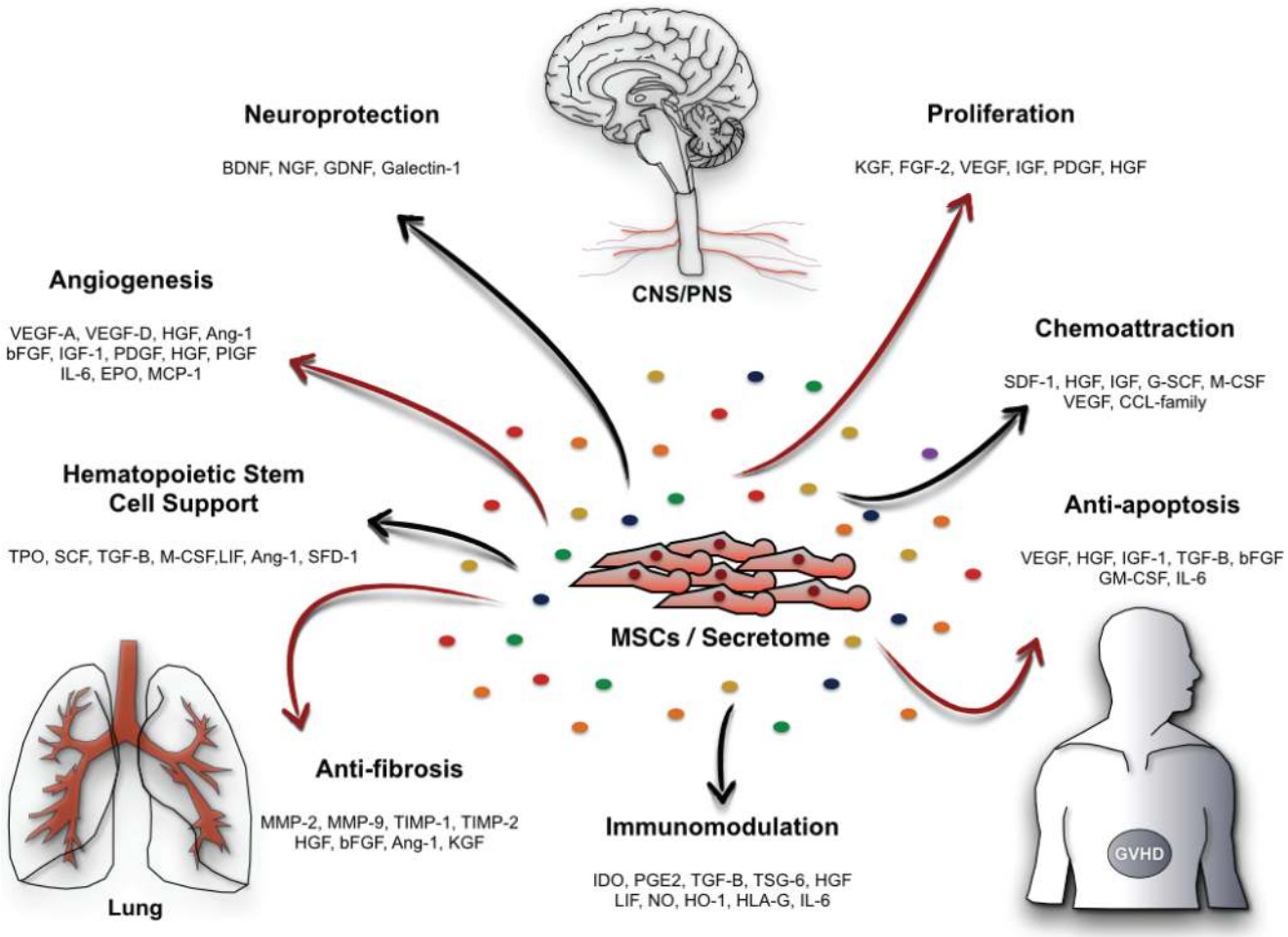



**Figure 18.4** MSCs exert most of their therapeutic actions through their secretome. This is carried out by a number of proteins involved in the regulation of phenomena such as neuroprotection, proliferation, chemoattraction, inhibition of apoptosis, immunomodulation, stromal cells support, and angiogenesis.

## References


1 J. Ankrum and J.M. Karp. Mesenchymal stem cell therapy: two steps forward, one step back. *Trends Mol Med* 2010;**16**(5):203–209.
2 M.E. Bernardo, F. Locatelli, and W.E. Fibbe. Mesenchymal stromal cells. *Ann N Y Acad Sci* 2009;**1176**:101–117.
3 T.L. Bonfield, M.T. Koloze, D.P. Lennon, and A.I. Caplan. Defining human mesenchymal stem cell efficacy *in vivo*. *J Inflamm (Lond)* 2010;**7**:51.
4 Y. Chen, J.Z. Shao, L.X. Xiang, *et al.* Mesenchymal stem cells: a promising candidate in regenerative medicine. *Int J Biochem Cell Biol* 2008;**40**(5):815–820.
5 A. Uccelli, F. Benvenuto, A. Laroni, and D. Giunti. Neuroprotective features of mesenchymal stem cells. *Best Pract Res Clin Haematol* 2011;**24**(1):59–64.
6 A. Uccelli, A. Laroni, and M.S. Freedman. Mesenchymal stem cells for the treatment of multiple sclerosis and other neurological diseases. *Lancet Neurol* 2011;**10**(7):649–656.
7 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.
8 A.J. Friedenstein, U.F. Deriglasova, N.N. Kulagina, *et al.* Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol* 1974;**2**(2):83–92.
9 P.A. Zuk, M. Zhu, P. Ashjian, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**(12):4279–4295.
10 P.A. Zuk, M. Zhu, H. Mizuno, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**(2):211–228.
11 S. Gronthos, M. Mankani, J. Brahim, *et al.* Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2000;**97**(25):13625–13630.
12 S. Shi and S. Gronthos. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003;**18**(4):696–704.
13 Y. Fukuchi, H. Nakajima, D. Sugiyama, *et al.* Human placenta-derived cells have mesenchymal stem/progenitor cell potential. *Stem Cells* 2004;**22**(5):649–658.
14 M.H. Abumaree, M.A. Al Jumah, B. Kallionis, *et al.* Phenotypic and functional characterization of mesenchymal stem cells from chorionic villi of human term placenta. *Stem Cell Rev* 2013;**9**(1):16–31.
15 A. Erices, P. Conget, and J.J. Minguell. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;**109**(1):235–242.
16 H.S. Wang, S.C. Hung, S.T. Peng, *et al.* Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004;**22**(7):1330–1337.
17 M.L. Weiss and D.L. Troyer. Stem cells in the umbilical cord. *Stem Cell Rev* 2006;**2**(2):155–162.
18 G. Paul, I. Ozen, N.S. Christophensen, *et al.* The adult human brain harbors multipotent perivascular mesenchymal stem cells. *PLoS One* 2012;**7**(4):e35577.
19 R. Abdi, P. Florina, C.N. Adra, *et al.* Immunomodulation by mesenchymal stem cells: a potential therapeutic strategy for type 1 diabetes. *Diabetes* 2008;**57**(7):1759–1767.
20 K. English, A. French, and K.J. Wood. Mesenchymal stromal cells: facilitators of successful transplantation? *Cell Stem Cell* 2010;**7**(4):431–442.
21 N. Joyce, G. Annett, L. Wirthlin, *et al.* Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regen Med* 2010;**5**(6):933–946.
22 S. Jung, F.G. Teixeira, K.M. Panchalingam, *et al.* Potential therapeutic properties of human mesenchymal stem cells. *J Clin Stud* 2012;**4**(6):36–41.
23 S.R. Daher, B.H. Johnstone, D.G. Phinney, and K.L. March. Adipose stromal/stem cells: basic and translational advances: the IFATS collection. *Stem Cells* 2008;**26**(10):2664–2665.
24 D.G. Phinney and I. Isakova. Plasticity and therapeutic potential of mesenchymal stem cells in the nervous system. *Curr Pharm Des* 2005;**11**(10):1255–1265.
25 D.J. Prockop, S.A. Azizi, D. Colter, *et al.* Potential use of stem cells from bone marrow to repair the extracellular matrix and the central nervous system. *Biochem Soc Trans* 2000;**28**(4):341–345.
26 J.W. Lee, X. Fang, A. Krasnodembskaya, *et al.* Concise review: mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells* 2011;**29**(6):913–919.
27 F. Baron and R. Storb. Mesenchymal stromal cells: a new tool against graft-versus-host disease? *Biol Blood Marrow Transplant* 2012;**18**(6):822–840.
28 M. Francois and J. Galipeau. New insights on translational development of mesenchymal stromal cells for suppressor therapy. *J Cell Physiol* 2012;**227**(11):3535–3538.
29 N. Hu, H. Wu, C. Xue, *et al.* Long-term outcome of the repair of 50 mm long median nerve defects in rhesus monkeys with marrow mesenchymal stem cells-containing, chitosan-based tissue engineered nerve grafts. *Biomaterials* 2013;**34**(1):100–111.
30 X. Wei, L. Zhao, J. Zhong, *et al.* Adipose stromal cells-secreted neuroprotective media against neuronal apoptosis. *Neurosci Lett* 2009;**462**(1):76–79.
31 O. Honmou, R. Onodera, M. Sasaki, *et al.* Mesenchymal stem cells: therapeutic outlook for stroke. *Trends Mol Med* 2012;**18**(5):292–297.
32 G. Constantin, S. Marconi, B. Rossi, *et al.* Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. *Stem Cells* 2009;**27**(10):2624–2635.
33 M. Cristofanilli, V.K. Harris, A. Zigelbaum, *et al.* Mesenchymal stem cells enhance the engraftment and myelinating ability of allogeneic oligodendrocyte progenitors in dysmyelinated mice. *Stem Cells Dev* 2011;**20**(12):2065–2076.
34 L. Cova, M.T. Armentero, E. Zennaro, *et al.* Multiple neurogenic and neurorescue effects of human mesenchymal stem cell after transplantation in an experimental model of Parkinson's disease. *Brain Res* 2010;**1311**:12–27.
35 P. Erba, G. Terenghi, and P.J. Kingham. Neural differentiation and therapeutic potential of adipose tissue derived stem cells. *Curr Stem Cell Res Ther* 2010;**5**(2):153–160.
36 D. Arboleda, S. Forostyak, P. Jendelova, *et al.* Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury. *Cell Mol Neurobiol* 2011;**31**(7):1113–1122.
37 J.H. Park, D.Y. Kim, I.Y. Sung, *et al.* Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. *Neurosurgery* 2012;**70**(5):1238–1247, discussion 1247.

38 F.G. Teixeira, M.M. Carvalho, N. Sousa, and A.J. Salgado. Mesenchymal stem cells secretome: a new paradigm for central nervous system regeneration? *Cell Mol Life Sci* 2013;**70**(20):3871–3882.

39 M.F. Pittenger, A.M. Mackay, S.C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999; **284**(5411):143–147.

40 D.J. Maltman, S.A. Hardy, and S.A. Przyborski. Role of mesenchymal stem cells in neurogenesis and nervous system repair. *Neurochem Int* 2011;**59**(3):347–356.

41 G. Chamberlain, J. Fox, B. Ashton, and J. Middleton. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007;**25**(11):2739–2749.

42 M. Gnecchi, H. He, O.D. Liang, *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;**11**(4):367–368.

43 A.J. Salgado, R.L. Reis, N. Sousa, and J.M. Gimble. Adipose tissue derived stem cells secretome: soluble factors and their roles in regenerative medicine. *Curr Stem Cell Res Ther* 2010;**5**(2):103–110.

44 M.M. Carvalho, F.G. Teixeira, R.L. Reis, *et al.* Mesenchymal stem cells in the umbilical cord: phenotypic characterization, secretome and applications in central nervous system regenerative medicine. *Curr Stem Cell Res Ther* 2011;**6**(3):221–228.

45 C.A. Ribeiro, A.J. Salgado, J.S. Fraga, *et al.* The secretome of bone marrow mesenchymal stem cells-conditioned media varies with time and drives a distinct effect on mature neurons and glial cells (primary cultures). *J Tissue Eng Regen Med* 2011;**5**(8):668–672.

46 J.S. Fraga, N.A. Silva, A.S. Lourenço, *et al.* Unveiling the effects of the secretome of mesenchymal progenitors from the umbilical cord in different neuronal cell populations. *Biochimie* 2013;**95**(12):2297–2303.

47 F.G. Teixeira, M.M. Carvalho, A. Neves-Carvalho, *et al.* Secretome of mesenchymal progenitors from the umbilical cord acts as modulator of neural/glial proliferation and differentiation. *Stem Cell Rev* 2015;**11**(2):288–297.

48 T. Meyerrose, S. Olson, S. Pontow, *et al.* Mesenchymal stem cells for the sustained *in vivo* delivery of bioactive factors. *Adv Drug Deliv Rev* 2010;**62**(12):1167–1174.

49 L. Chen, E.E. Tredget, P.Y. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS One* 2008;**3**(4): e1886.

50 G.J. Block, S. Ohkouchi, F. Fung, *et al.* Multipotent stromal cells are activated to reduce apoptosis in part by upregulation and secretion of stanniocalcin-1. *Stem Cells* 2009;**27**(3):670–681.

51 Y. Shi, G. Hu, J. Su, *et al.* Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. *Cell Res* 2010;**20** (5):510–518.

52 J.A. Kode, S. Mukherjee, M.V. Joglekar, and A.A. Hardikar Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* 2009;**11**(4):377–391.

53 A.I. Caplan and J.E. Dennis. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006;**98**(5):1076–1084.

54 J. Rehman, D. Traktuev, J. Li, *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;**109**(10):1292–1298.

55 N. Nakano, Y. Nakai, T.B. Seo, *et al.* Characterization of conditioned medium of cultured bone marrow stromal cells. *Neurosci Lett* 2010;**483**(1):57–61.

56 S.R. Baglio, D.M. Pegtel, and N. Baldini. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012;**3**:359.

57 D. Drago, C. Cossetti, N. Iraci, *et al.* The stem cell secretome and its role in brain repair. *Biochimie* 2013;**95**(12):2271–2285.

58 G. Camussi, M.C. Deregibus, and C. Tetta. Paracrine/endocrine mechanism of stem cells on kidney repair: role of microvesicle-mediated transfer of genetic information. *Curr Opin Nephrol Hypertens* 2010;**19**(1):7–12.

59 J. Ratajczak, M. Wysoczynski, F. Hayek, *et al.* Membrane-derived microvesicles: important and underappreciated mediators of cell-to-cell communication. *Leukemia* 2006;**20**(9):1487–1495.

60 M.Z. Ratajczak, M. Kucia, T. Jadczyk, *et al.* Pivotal role of paracrine effects in stem cell therapies in regenerative medicine: can we translate stem cell-secreted paracrine factors and microvesicles into better therapeutic strategies? *Leukemia* 2012;**26**(6):1166–1173.

61 C. Cossetti, J.A. Smith, N. Iraci, *et al.* Extracellular membrane vesicles and immune regulation in the brain. *Front Physiol* 2012;**3**:117.

62 H. Valadi, K. Ekström, A. Bossios, *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;**9**(6):654–659.

63 R.C. Lai, S.S. Tan, B.J. Teh, *et al.* Proteolytic potential of the MSC Exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int J Proteomics* 2012;**2012**:971907.

64 C. Harding, J. Heuser, and P. Stahl. Receptor-mediated endocytosis of transferrin and recycling of the transferrin receptor in rat reticulocytes. *J Cell Biol* 1983;**97**(2):329–339.

65 B. Yu, X. Zhang, and X. Li. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci* 2014;**15**(3):4142–4157.

66 R.W. Yeo, R.C. Lai, B. Zhang, *et al.* Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013;**65**(3):336–341.

67 M. Eldh, K. Ekström, H. Valadi, *et al.* Exosomes communicate protective messages during oxidative stress; possible role of exosomal shuttle RNA. *PLoS One* 2010;**5**(12):e15353.

68 R.C. Lai, R.W. Yeo, K.H. Tan, and S.K. Lim. Exosomes for drug delivery – a novel application for the mesenchymal stem cell. *Biotechnol Adv* 2013;**31**(5):543–551.

69 D.M. Pegtel, K. Cosmopoulos, D.A. Thorley-Lawson, *et al.* Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A* 2010;**107**(14):6328–6333.

70 B. Zhang, Y. Yin, R.C. Lai, *et al.* Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* 2014;**23**(11):1233–1244.

71 F. Arslan, R.C. Lai, M.B. Smeets, *et al.* Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013;**10**(3):301–312.

72 T. Katsuda, R. Tsuchiya, N. Kosaka, *et al.* Human adipose tissue-derived mesenchymal stem cells secrete functional neprilysin-bound exosomes. *Sci Rep* 2013;**3**:1197.

73 R.C. Lai, F. Arslan, M.M. Lee, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**(3):214–222.

74 T. Li, Y. Yan, B. Wang, *et al.* Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013;**22**(6):845–854.

75 L.A. Reis, F.T. Borges, M.J. Simoes, *et al.* Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats. *PLoS One* 2012;**7**(9):e44092.

76 C.Y. Tan, R.C. Lai, W. Wong, *et al.* Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 2014;**5**(3):76.

77 Y. Zhou, H. Xu, W. Xu, *et al.* Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis *in vivo* and *in vitro*. *Stem Cell Res Ther* 2013;**4**(2):34.

78 H.K. Skalnikova, J. Motlik, S.J. Gadher, and H. Kovarova. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. *Proteomics* 2011;**11**(4):691–708.

79 H.K. Skalnikova. Proteomic techniques for characterisation of mesenchymal stem cell secretome. *Biochimie* 2013;**95**(12):2196–2211.

80 M.H. Maurer. Proteomic definitions of mesenchymal stem cells. *Stem Cells Int* 2011;**2011**:704256.

81 T. Kuboki, F. Kantawong, R. Burchmore, *et al.* 2D-DIGE proteomic analysis of mesenchymal stem cell cultured on the elasticity-tunable hydrogels. *Cell Struct Funct* 2012;**37**(2):127–139.

82 G. Li, C.Y. Chan, H. Wang, and H.F. Kung. Proteomic analysis of human mesenchymal stem cells. *Methods Mol Biol* 2011;**698**:443–457.

83 S.P. Gygi and R. Aebersold. Absolute quantitation of 2-D protein spots. *Methods Mol Biol* 1999;**112**:417–421.

84 P. Scherp, G. Ku, L. Coleman, and K. Sergeant. Gel-based and gel-free proteomic technologies. *Methods Mol Biol* 2011;**702**:163–90.

85 X. Zhang, A. Fang, C.P. Riley, *et al.* Multi-dimensional liquid chromatography in proteomics – a review. *Anal Chim Acta* 2010;**664**(2):101–113.

86 M. Groessl, H. Luksch, A. Rosen-Wolff, *et al.* Profiling of the human monocytic cell secretome by quantitative label-free mass spectrometry identifies stimulus-specific cytokines and proinflammatory proteins. *Proteomics* 2012;**12**(18):2833–2842.

87 M. Gilar, P. Olivova, A.E. Daly, and J.C. Gebler. Two-dimensional separation of peptides using RP-RP-HPLC system with different pH in first and second separation dimensions. *J Sep Sci* 2005;**28**(14):1694–1703.

88 F. Yang, Y. Shen, D.G. Camp, and R.D. Smith. High-pH reversed-phase chromatography with fraction concatenation for 2D proteomic analysis. *Expert Rev Proteomics* 2012;**9**(2):129–134.

89 E. Capra, R. Beretta, V. Parazzi, *et al.* Changes in the proteomic profile of adipose tissue-derived mesenchymal stem cells during passages. *Proteome Sci* 2012;**10**(1):46.

90 W. Zhu, J.W. Smith, and C.M. Huang. Mass spectrometry-based label-free quantitative proteomics. *J Biomed Biotechnol* 2010;**2010**:840518.

91 S.P. Blaber, R.A. Webster, C.J. Hill, *et al.* Analysis of *in vitro* secretion profiles from adipose-derived cell populations. *J Transl Med* 2012;**10**:172.

92 K.M. Mrozik, P.S. Zilm, C.J. Bagley, *et al.* Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins. *Stem Cells Dev* 2010;**19**(10):1485–1499.

93 V. Lange, P. Picotti, B. Domon, and R. Aebersold. Selected reaction monitoring for quantitative proteomics: a tutorial. *Mol Syst Biol* 2008.**4**:222.

94 S.E. Ong, B. Blagoev, I. Kratchmarova, *et al.* Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol Cell Proteomics* 2002;**1**(5):376–386.

95 L.P. Kristensen, L. Chen, M.O. Nielsen, *et al.* Temporal profiling and pulsed SILAC labeling identify novel secreted proteins during *ex vivo* osteoblast differentiation of human stromal stem cells. *Mol Cell Proteomics* 2012;**11**(10):989–1007.

96 S.P. Gygi, B. Rist, S.A. Gerber, *et al.* Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat Biotechnol* 1999;**17**(10):994–999.

97 P.L. Ross, Y.N. Huang, J.N. Marchese, *et al.* Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol Cell Proteomics* 2004;**3**(12):1154–1169.

98 N. Verma, A.W. Rettenmeier, and S. Schmitz-Spanke. Recent advances in the use of *Sus scrofa* (pig) as a model system for proteomic studies. *Proteomics* 2011;**11**(4):776–793.

99 J.C. Voltarelli, H. Ahmed, E.J. Paton, *et al.* Beneficial effect of intravenous lidocaine in cutaneous chronic graft-versus-host disease secondary to donor lymphocyte infusion. *Bone Marrow Transplant* 2001;**28**(1):97–99.

100 J.C. Voltarelli and J. Ouyang. Hematopoietic stem cell transplantation for autoimmune diseases in developing countries: current status and future prospectives. *Bone Marrow Transplant* 2003;**32**(Suppl 1):S69–71.

101 J.Y. Weng, X. Du, S.X. Geng, *et al.* Mesenchymal stem cell as salvage treatment for refractory chronic GVHD. *Bone Marrow Transplant* 2010;**45**(12):1732–1740.

102 D. Wolf, M. von Lilienfeld-Toal, A.M. Wolf, *et al.* Novel treatment concepts for graft-versus-host disease. *Blood* 2012;**119**(1):16–25.

103 H.J. Deeg. How I treat refractory acute GVHD. *Blood* 2007;**109**(10):4119–4126.

104 O. Ringden and K. Le Blanc. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. *Best Pract Res Clin Haematol* 2011;**24**(1):65–72.

105 O. Ringden and A. Keating. Mesenchymal stromal cells as treatment for chronic GVHD. *Bone Marrow Transplant* 2011;**46**(2):163–164.

106 D. Maher, L. Blanc, and M. Raviglione. WHO policies for tuberculosis control. *Lancet* 2004.**363**(9424): 1911.

107 J. Kurtzberg, S. Prockop, P. Teira, *et al.* Allogeneic human mesenchymal stem cell therapy (remestemcel-L, Prochymal) as a rescue agent for severe refractory acute graft-versus-host disease in pediatric patients. *Biol Blood Marrow Transplant* 2014;**20**(2):229–235.

108 C.A. Ribeiro, J.S. Fraga, M. Grãos, *et al.* The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations. *Stem Cell Res Ther* 2012;**3**(3):18.

109 A.J. Salgado, J.S. Fraga, A.R. Mesquita, *et al.* Role of human umbilical cord mesenchymal progenitors conditioned media in neuronal/glial cell densities, viability, and proliferation. *Stem Cells Dev* 2010;**19**(7):1067–1074.

110 L. Crigler, R.C. Robey, A. Asawachaicham, *et al.* Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Exp Neurol* 2006;**198**(1):54–64.

111 W. Gu, F. Zhang, Q. Xue, *et al.* Transplantation of bone marrow mesenchymal stem cells reduces lesion volume and induces axonal regrowth of injured spinal cord. *Neuropathology* 2010;**30**(3):205–217.

112 G.Z. Jin, S.J. Cho, Y.S. Lee, *et al.* Intrastriatal grafts of mesenchymal stem cells in adult intact rats can elevate tyrosine hydroxylase expression and dopamine levels. *Cell Biol Int* 2010;**34**(1):135–140.

113 M.K. McCoy, T.N. Martinez, K.A. Ruhn, *et al.* Autologous transplants of adipose-derived adult stromal (ADAS) cells afford dopaminergic neuroprotection in a model of Parkinson's disease. *Exp Neurol* 2008;**210**(1):14–29.

114 Y.S. Fu, Y.C. Cheng, M.Y. Lin, *et al.* Conversion of human umbilical cord mesenchymal stem cells in Wharton's jelly to dopaminergic neurons *in vitro*: potential therapeutic application for Parkinsonism. *Stem Cells* 2006;**24**(1):115–124.

115 T. Lopatina, N. Kalinina, M. Karagyaur, *et al.* Adipose-derived stem cells stimulate regeneration of peripheral nerves: BDNF secreted by these cells promotes nerve healing and axon growth de novo. *PLoS One* 2011;**6**(3):e17899.

116 C.C. Yang, Y.H. Shih, M.H. Ko, *et al.* Transplantation of human umbilical mesenchymal stem cells from Wharton's jelly after complete transection of the rat spinal cord. *PLoS One* 2008;**3**(10):e3336.

117 K. Wakabayashi, A. Nagai, A.M. Sheikh, *et al.* Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J Neurosci Res* 2010;**88**(5):1017–1025.

118 S.H. Koh, K.S. Kim, M.R. Choi, *et al.* Implantation of human umbilical cord-derived mesenchymal stem cells as a neuroprotective therapy for ischemic stroke in rats. *Brain Res* 2008;**1229**:233–248.

119 A.J. Shang, S.Q. Hong, Q. Xu, *et al.* NT-3-secreting human umbilical cord mesenchymal stromal cell transplantation for the treatment of acute spinal cord injury in rats. *Brain Res* 2011;**1391**:102–113.

120 M. Zurita and J. Vaquero. Bone marrow stromal cells can achieve cure of chronic paraplegic rats: functional and morphological outcome one year after transplantation. *Neurosci Lett* 2006;**402**(1–2):51–56.

121 C. Ide, Y. Nakai, N. Nakano, *et al.* Bone marrow stromal cell transplantation for treatment of sub-acute spinal cord injury in the rat. *Brain Res* 2010;**1332**:32–47.

122 J. Vaquero and M. Zurita. Bone marrow stromal cells for spinal cord repair: a challenge for contemporary neurobiology. *Histol Histopathol* 2009;**24**(1):107–116.

123 K.T. Wright, W. El Masri, A. Osman, *et al.* Bone marrow stromal cells stimulate neurite outgrowth over neural proteoglycans (CSPG), myelin associated glycoprotein and Nogo-A. *Biochem Biophys Res Commun* 2007;**354**(2):559–566.

124 P. Lu, L.L. Jones, and M.H. Tuszynski. BDNF-expressing marrow stromal cells support extensive axonal growth at sites of spinal cord injury. *Exp Neurol* 2005;**191**(2):344–360.

125 B. Neuhuber, B. Timothy Himes, J.S. Shumsky, *et al.* Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. *Brain Res* 2005;**1035**(1):73–85.

126 H.W. Park, M.J. Lim, H. Jung, *et al.* Human mesenchymal stem cell-derived Schwann cell-like cells exhibit neurotrophic effects, via distinct growth factor production, in a model of spinal cord injury. *Glia* 2010;**58**(9):1118–1132.

127 L. Zhang, H.T. Zhang, S.Q. Hong, *et al.* Cografted Wharton's jelly cells-derived neurospheres and BDNF promote functional recovery after rat spinal cord transection. *Neurochem Res* 2009;**34**(11):2030–2039.

128 D. Cantinieaux, R. Quertainmont, S. Blacher, *et al.* Conditioned medium from bone marrow-derived mesenchymal stem cells improves recovery after spinal cord injury in rats: an original strategy to avoid cell transplantation. *PLoS One* 2013;**8**(8):e69515.

129 F. Saito, T. Nakatani, M. Iwase, *et al.* Administration of cultured autologous bone marrow stromal cells into cerebrospinal fluid in spinal injury patients: a pilot study. *Restor Neurol Neurosci* 2012;**30**(2):127–136.

130 S. Karamouzian, S.N. Nematollahi-Mahani, N. Nakhaee, and H. Eskandary. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. *Clin Neurol Neurosurg* 2012;**114**(7):935–939.

131 E.A. Pereira and T.Z. Aziz. Surgical insights into Parkinson's disease. *J R Soc Med* 2006;**99**(5):238–244.

132 W.C. Koller (ed.). *Handbook of Parkinson's Disease*, 3rd edn. Marcel Dekker: New York, 2003.

133 G. Cummins and R.A. Barker. What is the most promising treatment for Parkinson's disease: genes, cells, growth factors or none of the above? *Regen Med* 2012;**7**(5):617–621.

134 W. Dauer and S. Przedborski. Parkinson's disease: mechanisms and models. *Neuron*, 2003;**39**(6):889–909.

135 M.L. Weiss, S. Medicetty, A.R. Bledsoe, *et al.* Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* 2006;**24**(3):781–792.

136 A. Shintani, N. Nakao, K. Kakishita, and T. Itakura. Protection of dopamine neurons by bone marrow stromal cells. *Brain Res* 2007;**1186**:48–55.

137 O. Sadan, M. Bahat-Stromza, Y. Barhum, *et al.* Protective effects of neurotrophic factor-secreting cells in a 6-OHDA rat model of Parkinson disease. *Stem Cells Dev* 2009;**18**(8):1179–1190.

138 Y.J. Kim, H.J. Park, G. Lee, *et al.* Neuroprotective effects of human mesenchymal stem cells on dopaminergic neurons through anti-inflammatory action. *Glia* 2009;**57**(1):13–23.

139 F. Blandini, L. Cova, M.T. Armentero, *et al.* Transplantation of undifferentiated human mesenchymal stem cells protects against 6-hydroxydopamine neurotoxicity in the rat. *Cell Transplant* 2010;**19**(2):203–217.

140 F. Wang, T. Yasuhara, T. Shingo, *et al.* Intravenous administration of mesenchymal stem cells exerts therapeutic effects on parkinsonian model of rats: focusing on neuroprotective effects of stromal cell-derived factor-1α. *BMC Neurosci* 2010;**11**:52.

141 L. Danielyan, R. Schafer, A. von Ameln-Mayerhofer, *et al.* Therapeutic efficacy of intranasally delivered mesenchymal stem cells in a rat model of Parkinson disease. *Rejuvenation Res* 2011;**14**(1):3–16.

142 H.J. Park, J.Y. Shin, B.R. Lee, *et al.* Mesenchymal stem cells augment neurogenesis in the subventricular zone and enhance differentiation of neural precursor cells into dopaminergic neurons in the substantia nigra of a parkinsonian model. *Cell Transplant* 2012;**21**(8):1629–1640.

143 A. Björklund, C. Rosenblad, C. Winkler, and D. Kirik. Studies on neuroprotective and regenerative effects of GDNF in a partial lesion model of Parkinson's disease. *Neurobiol Dis* 1997;**4**(3–4):186–200.

144 Hyman, C., M. Hofer, Y.A. Barde, *et al.* BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. *Nature* 1991;**350**(6315):230–232.

145 L.F. Lin, D.H. Doherty, J.D. Lile, *et al.* GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. *Science* 1993;**260**(5111):1130–1132.

146 E. Mayer, S.B. Dunnett, R. Pellitteri, and J.W. Fawcett. Basic fibroblast growth factor promotes the survival of embryonic ventral mesencephalic dopaminergic neurons – I. *Effects in vitro. Neuroscience* 1993;**56**(2):379–388.

147 C. Hyman, S.B. Dunnett, R. Pellitteri, *et al.* Overlapping and distinct actions of the neurotrophins BDNF, NT-3, and NT-4/5 on cultured dopaminergic and GABAergic neurons of the ventral mesencephalon. *J Neurosci* 1994;**14**(1):335–347.

148 J. Noble, C.A. Munro, V.S. Prasad, and R. Midha. Analysis of upper and lower extremity peripheral nerve injuries in a population of patients with multiple injuries. *J Trauma* 1998;**45**(1):116–122.

149 V. Mukhatyar, L. Karumbaiah, J. Yeh, and R. Bellamkonda. Tissue engineering strategies designed to realize the endogenous regenerative potential of peripheral nerves. *Adv Mater* 2009;**21**(46): 4670–4679.

150 M.L. Groves, R. McKeon, E. Werner, *et al.* Axon regeneration in peripheral nerves is enhanced by proteoglycan degradation. *Exp Neurol* 2005;**195**(2):278–292.

151 X.H. Duan, L.N. Cheng, F. Zhang, *et al. In vivo* MRI monitoring nerve regeneration of acute peripheral nerve traction injury following mesenchymal stem cell transplantation. *Eur J Radiol* 2012;**81**(9):2154–2160.

152 J. Hu, Q.T. Zhu, X.L. Liu, *et al.* Repair of extended peripheral nerve lesions in rhesus monkeys using acellular allogenic nerve grafts implanted with autologous mesenchymal stem cells. *Exp Neurol* 2007;**204**(2):658–666.

153 G. Keilhoff and H. Fansa. Mesenchymal stem cells for peripheral nerve regeneration – a real hope or just an empty promise? *Exp Neurol* 2011;**232**(2):110–113.

154 J.Y. Lee, H.J. Jung, M.S. Alrashdan, *et al.* Peripheral nerve regeneration with cotransplantation of umbilical cord mesenchymal stem cells and Schwann cells in rat sciatic nerve defect. *Neural Regen Res* 2011;**6**(7):485–493.

155 G. Liu, Y. Cheng, S. Guo, *et al.* Transplantation of adipose-derived stem cells for peripheral nerve repair. *Int J Mol Med* 2011;**28** (4):565–572.

156 M. Tohill and G. Terenghi. Stem-cell plasticity and therapy for injuries of the peripheral nervous system. *Biotechnol Appl Biochem* 2004.**40**(Pt 1):17–24.

157 H.C. Pan, F.C. Cheng, C.J. Chen, *et al.* Post-injury regeneration in rat sciatic nerve facilitated by neurotrophic factors secreted by amniotic fluid mesenchymal stem cells. *J Clin Neurosci* 2007;**14** (11):1089–1098.

158 D.Y. Kim, Y.S. Choi, S.E. Kim, *et al. In vivo* effects of adipose-derived stem cells in inducing neuronal regeneration in sprague-dawley rats undergoing nerve defect bridged with polycaprolactone nanotubes. *J Korean Med Sci* 2014;**29**(Suppl 3): S183–S192.

159 S. Khalifian, K.A. Sarhane, M. Tammia, *et al.* Stem cell-based approaches to improve nerve regeneration: potential implications for reconstructive transplantation? *Arch Immunol Ther Exp (Warsz)* 2015;**63**(1):15–30.

160 S. Wrobel, S.C. Serra, S. Ribeiro-Samy, *et al.* In vitro evaluation of cell-seeded chitosan films for peripheral nerve tissue engineering. *Tissue Eng Part A* 2014;**20**(17–18):2339–2349.

161 M.F. Azari, L. Mathias, E. Ozturk, *et al.* Mesenchymal stem cells for treatment of CNS injury. *Curr Neuropharmacol* 2010;**8**(4):316–323.

162 M. Kassem, M. Kristiansen, and B.M. Abdallah. Mesenchymal stem cells: cell biology and potential use in therapy. *Basic Clin Pharmacol Toxicol* 2004;**95**(5):209–214.

163 L.S. Sasportas, R. Kasmieh, H. Wakimoto, *et al.* Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy. *Proc Natl Acad Sci U S A* 2009;**106** (12):4822–4827.

164 P. Semedo, C.G. Palasio, C.D. Oliveira, *et al.* Early modulation of inflammation by mesenchymal stem cell after acute kidney injury. *Int Immunopharmacol* 2009;**9**(6):677–682.

165 S. Wang, X. Qu, and R.C. Zhao. Mesenchymal stem cells hold promise for regenerative medicine. *Front Med* 2011;**5**(4):372–378.

166 J.S. Lee, J.M. Hong, G.J. Moon, *et al.* A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 2010;**28**(6):1099–1106.

CHAPTER 19

# The secretome of mesenchymal stem/stromal cells undergoing chondrogenic differentiation and those undergoing osteogenic or adipogenic differentiation

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## 19.1 Introduction to protein secretion and the analysis of secretomes

Secretomics is a recent proteomic approach that seeks to describe the secretome, considering it as the set of proteins secreted by a cell, tissue, or organism at any given time or under certain conditions. The term *secretome* was introduced for the first time by Tjalsma *et al.* in a study of those proteins secreted by *Bacillus subtillis* [1]. It is considered to be encoded by approximately 10% of the human genome [2,3]. The secretome constitutes an important class of proteins including components of the extracellular matrix (ECM) and regulatory molecules. These play essential roles in several physiological and pathophysiological processes, including cell signaling, differentiation, cell adhesion, angiogenesis, and apoptosis [4]. To date, the secretomes of mesenchymal stem/stromal cells (MSCs) and other types of stem cells have been explored [5], leading to the identification of several different molecules, including growth factors and the proteins binding to them, proinflammatory and anti-inflammatory cytokines, chemokines, ECM structural proteins, and remodeling enzymes.

Protein secretion is a common, complex, and well-controlled process. Normally, cells react to different stimuli and signals that they receive from their environment and consequently activate or inactivate the secretion of a number of bioactive molecules. A protein secretion balance is essential for normal cell function. Thus, imbalance or alterations in the protein secretion pattern/profile (the secretome) can be indicative of a pathologic condition [6]. Furthermore, the secretome also reflects the functional status of a cell in a given environment. Hence, secretomics is a very attractive approach for discovering novel diagnostic or prognostic biomarkers and also for putative therapeutic targets.

The proteins are secreted from the cells through two main secretory pathways: conventional and unconventional [7]. In the conventional secretory pathway, proteins with a signal peptide sequence are translocated from the ribosomes into the lumen of the endoplasmic reticulum, where they undergo post-translational modifications. Once these proteins have been correctly folded and glycosylated, they travel to the Golgi apparatus before they reach their destination – the extracellular space, the plasma membrane, or other target organelles.

The conventional pathways are further divided into constitutive secretion and regulated exocytosis. In the constitutive secretion pathway, proteins are carried continuously by vesicular carriers from the Golgi apparatus to the plasma membrane or endosomes. In regulated exocytosis, proteins are sorted and stored in granules, which can reach the plasma membrane in three ways: direct fusion with the membrane, transport in small vesicles to the membrane, or fusion with each other on the cell surface [8].

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Unconventional secretory pathways are used by proteins with or without signal peptide sequences. Most of the cytoplasmic and nuclear proteins avoid the endoplasmic reticulum–Golgi system and are secreted by an unconventional transport pathway [9]. Unconventional secretion takes place using specialized membrane vesicles or by employing direct protein translocation across the membrane. Interleukin (ILIL)-1β, fibroblast growth factor (FGF), and galectins are exported through the unconventional transport pathways.

## 19.2 Analysis of mesenchymal stem/stromal cell secretomes using proteomic approaches

Proteomic studies on MSC secretomes have greatly increased in recent years owing to the improvements in proteomic platforms and mass spectrometry instrumentation. Although the secretome is quite complex because of the difficulties in its collection and preparation for protein analysis, more than 35 studies have focused on the characterization of the MSC secretome in the past few years. Table 19.1 summarizes the objective and technological strategy followed of each of these studies. All these publications denote the growing interest in the MSCs' secretome, which is largely due to the potential applications of these cells in regenerative medicine.

### 19.2.1 Approaches to obtaining secretome samples

Currently, secretome studies can be performed in three different ways: *in vitro*, using cell culture systems, *ex vivo*, *by* analyzing the secretome of tissue explants without isolating the cells, or *in vivo*, using animal models [48].

#### 19.2.1.1 *In vitro* secretome studies

These involve first the expansion of the cells in a serum (fetal bovine serum (FBS))-supplemented medium. Then the cells are extensively washed with sterile phosphate-buffered saline to remove FBS-derived proteins before incubating them in a serum-free medium. In these type of preparations FBS proteins are abundant and can mask the proteins secreted by the cells, affecting the separation and detection of proteins that are secreted in low amounts. The elimination of serum from the cell culture reduces sample complexity and also increases reproducibility. However, many cell lines are sensitive to serum-free media, and serum starvation can lead to an increase in cell death and the subsequent release of intracellular proteins. Additionally, the length of incubation in this type of experiment can vary from 12–24 h to 4–7 days and depends on the cell type involved.

#### 19.2.1.2 *Ex vivo* secretome studies

When the experiments are carried out under *ex vivo* conditions fresh tissue explants are incubated in serum-free medium for 12–24 h followed by the collection of the conditioned medium (CM) for protein identification. This approach has been successfully employed to study the secretome of human cartilage explants [49,50] and human adipose tissue [46]. However, *ex vivo* approaches demand careful sample handling and culturing to avoid false-positive results due to serum protein contaminants, which can remain inside or adhere to the surface of the tissue explants.

#### 19.2.1.3 *In vivo* secretome studies

These involve the analysis of body fluids, including serum, synovial fluid, urine, or interstitial fluid, that contain those proteins secreted by the tissues and cells in their native microenvironment. In this approach the body fluids are collected using microdialysis devices and capillary ultrafiltration probes [48]. The proteins are then concentrated and analyzed directly by mass spectrometry. In this way sample handling is reduced. However, *in vivo* secretome analysis has a number of issues that should be taken into account, including the high dynamic range of this type of sampling (which hampers the detection of low-abundance proteins), the possibility of contamination with blood-derived proteins, or difficulty knowing the relative contribution of a particular cell or tissue to the specific body fluid proteome [48].

### 19.2.2 Experimental strategies for *in vitro* secretome analysis of mesenchymal stem/stromal cells

Secretome analysis of MSCs is most commonly performed under *in vitro* conditions because of the low levels of secreted proteins in tissue explants or body fluids. It involves several steps, including MSC isolation, MSC expansion in a serum-containing culture medium, collection of CM, protein extraction, and proteomic analysis (Figure 19.1).

#### 19.2.2.1 Isolation of mesenchymal stem/stromal cells for proteomic analysis

MSCs can be isolated from a variety of human tissues, including, among others, bone marrow [14], adipose tissue [15], muscle [16], trabecular bone [17], and synovial membrane [18]. After harvesting the tissue, MSCs are usually extracted from the tissue by enzymatic digestion and expanded in monolayer cultures in order to obtain a sufficient number for the analyses. Classical expansion protocols involve a basic medium such as Dulbeccco's modified Eagle medium (DMEM) containing 10% FBS and cultured at 37 °C in a controlled atmosphere with 5% $CO_2$. After the initial culture phase the cells can be stimulated with different factors or differentiated to other cell types, such as chondrocytes, adipocytes, or osteoblasts. For example, to induce chondrogenic differentiation *in vitro*, cells are cultured in a three-dimensional culture system as pellets in the presence of specific growth factors that include transforming growth factor-β (TGF-β) and dexamethasone [19,20].

**Table 19.1** Studies of the secretomes of MSCs using proteomic techniques.

| Origin | MSC source | Cell treatment | Conditioning (incubation) time[a] | Sample type | Proteomic technique | Reference |
|---|---|---|---|---|---|---|
| Human | BM-MSCs/ MIAMI cells | Comparative analysis of BM-MSCs (expanded in 21% $O_2$) vs MIAMI cells (expanded in 3% $O_2$) | Not mentioned | Complete CM | Bio-Plex™ cytokine assay | [10] |
| Rat | ADSCs | None | 24 h | Complete CM (10% serum) | cDNA microarray/ELISA | [11] |
| Rat | BM-MSCs | None | 24 h | Complete CM | ELISA | [11] |
| Rat | BM-MSCs | *GATA4* gene transduction | 24 h | Serum-free CM | ELISA | [12] |
| Mouse | BM-MSCs | Effects of MSC secretome on T-cell differentiation | 4 h | CM from MSCs + T-cell co-culture | ELISA | [13] |
| Human | ADSCs | bFGF, EGF, and LPS | 12 days | Complete CM (10% serum) | ELISA | [14] |
| Human | ADSCs | Tumor necrosis factor | 24 h | Commercial medium | ELISA | [15] |
| Human | ADSCs | Hypoxia (1% and 21% $O_2$) | 72 h | Complete CM (5% serum) | ELISA | [16] |
| Human | BM-MSCs | Inflammatory response in Gaucher disease | 24 h | Serum-free CM | ELISA/RayBio® cytokine antibody array | [17] |
| Human | BM-MSCs | Hypoxia | 7 days | Complete CM | Luminex® antibody bead-based assay | [10] |
| Mouse | BM-MSCs | Effects of MSC secretome on T-cell proliferation and cytokine production | 48 h | Complete CM | Luminex antibody bead-based assay | [18] |
| Human | UCMSCs | None | 24, 48, 72, 96 h | Serum-free CM | Luminex antibody bead-based assay | [19] |
| Human | ADSCs | None | 24, 48, 72, 96 h | Serum-free CM | Luminex antibody bead-based assay | [19] |
| Human | BM-MSCs | Osteogenic differentiation | 48 h | Complete CM | RayBio angiogenesis antibody array/ELISA | [20] |
| Human | BM-MSCs | Serum starvation to produce angiogenic factors | 24 h | Serum-free CM | RayBio angiogenesis antibody array/ELISA | [21] |
| Human | BM-MSCs | None | Not mentioned | CM | RayBio cytokine antibody array | [22] |
| Rat | BM-MSCs | Chondrogenic and osteogenic differentiation | Not mentioned | Complete CM | RayBio cytokine antibody array | [23] |
| Mouse | BM-MSCs | MSC CM suppression of immunoglobulin production by plasma cells | Not mentioned | Complete CM | RayBio cytokine antibody array | [24] |
| Rat | ADSCs | None | 24 h | Serum-free CM | RayBio cytokine antibody array/ELISA | [25] |
| Human | Adipose tissue | None | 24 h | Complete CM | RayBio cytokine antibody array/ELISA | [26] |
| Human | MSCs derived from HuES9. E1 cell line | None | 3 days | Serum-free CM | RayBio cytokine antibody array/LC-MS/MS | [27] |
| Human | UCMSCs | None | 4 days | Serum-free CM | RayBio cytokine array | [28] |
| Mouse | BM-MSCs | None | 42 h | Serum-free CM | LC–MS/MS | [29] |
| Mouse | BM-MSCs | None | 42 h | Cell lysate (search for potentially secreted proteins) | LC–MS/MS | [30] |

**Table 19.1** (*Continued*)

| Origin | MSC source | Cell treatment | Conditioning (incubation) time[a] | Sample type | Proteomic technique | Reference |
|---|---|---|---|---|---|---|
| Human | BM-MSCs | Osteogenic differentiation | 12, 16 h | Serum-free CM | LC–MS/MS | [31] |
| Human | BM-MSCs | Osteogenic differentiation | 24 h | Serum-free CM | LC–MS/MS | [32] |
| Human | BM-MSCs | Osteogenic differentiation | 24 h | Serum-free CM | LC–MS/MS | [33] |
| Mouse + Human | BM-MSCs | Neural differentiation | 24 h | Serum-free CM | LC–MS/MS | [12] |
| Human | ADSCs | TNF | 48 h | Serum-free CM | LC–MS/MS | [34] |
| Human | BM-MSCs | Effects of MSC secretome on pulmonary epithelial repair | 24 h | Complete CM (0.2% FBS) | LC–MS/MS | [35] |
| Equine | BM-MSCs | Chondrogenic and osteogenic differentiation | 4 days | Serum-free CM | LC–MS/MS/Human Angiogenesis Proteome Profiler™ antibody array | [36] |
| Human | BM-MSCs | None | 24 h | Microvesicles isolated from serum-free CM | 1D SDS-PAGE + LC–MS/MS | [59] |
| Human | ADSCs | Adipogenic and osteogenic differentiation | 6 h | Serum free CM | 1D SDS-PAGE + LC–MS/MS | [37] |
| Human | UCMSCs | Chondrogenic differentiation | 12 h | Serum free CM | 1D SDS-PAGE + LC–MS/MS | [38] |
| Human | BM-MSCs | NCT, NCA, and chondrogenic differentiation | 21 days | Complete CM | 1D SDS-PAGE + LC–MS/MS | [39] |
| Rat | Adipose tissue | Insulin | 48 h | Serum-free CM | 2D LC–MS/MS | [40] |
| Mouse | A549 cells (MSCs) | None | 48 h | Complete CM | 2-DE + LC–MS/MS | [41] |
| Human | ADSCs | Adipogenic differentiation | 16 h | Serum-free CM | 2-DE + MS/MS | [42] |
| Mouse | BM-MSCs | bFGF | 32 h | Serum-free CM | SILAC + 1D SDS-PAGE + LC–MS/MS | [43] |
| Human | hMSC-TERT cells (BM-MSCs) | Osteogenic differentiation | 18 h | Serum-free CM | SILAC + 1D SDS-PAGE + LC–MS/MS | [44] |
| Human | BM-MSCs | Chondrogenic differentiation | 24 h | Serum-free CM | SILAC + 1D SDS-PAGE + LC–MS/MS | [45] |
| Human | Adipose tissue | None | 48–114 h (72 h for SILAC) | Serum-free CM | SILAC + 1D SDS-PAGE + LC–MS/MS/SELDI | [46] |
| Mouse | 3T3-L1 preadipocytes | Adipogenic differentiation | 18 h at the end of 0, 1, 3, 5, and 7 days | Serum-free CM | SILAC + LC–MS/MS | [47] |

CM: conditioned medium; BM-MSCs: bone-marrow-derived mesenchymal stem/stromal cells; ADSCs: adipose-tissue-derived stem/stromal cells; UCMSCs: umbilical cord mesenchymal stem/stromal cells; MIAMI: marrow-isolated adult multilineage inducible (cells). 1D: one-dimensional; 2D: two-dimensional; 2-DE: two-dimensional electrophoresis; bFGF: basic FGF; EGF: epidermal growth factor; ELISA: enzyme-linked immunosorbent assay; hMSC-TERT: human telomerase reverse transcriptase transduced MSC line; LC: liquid chromatography; LPS: lipopolysaccharide; MS/MS: tandem mass spectrometry; NCA: notochordal[a] cells in alginate tissue; NCT: notochordal[a] cells in native tissue; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SELDI: surface-enhanced laser desorption/ionization; SILAC: stable isotopic labeling by amino acids in cell culture; TNF: tumor necrosis factor.

[a] In most vertebrates the notochord becomes the nucleus pulposus of the intervertebral disc.

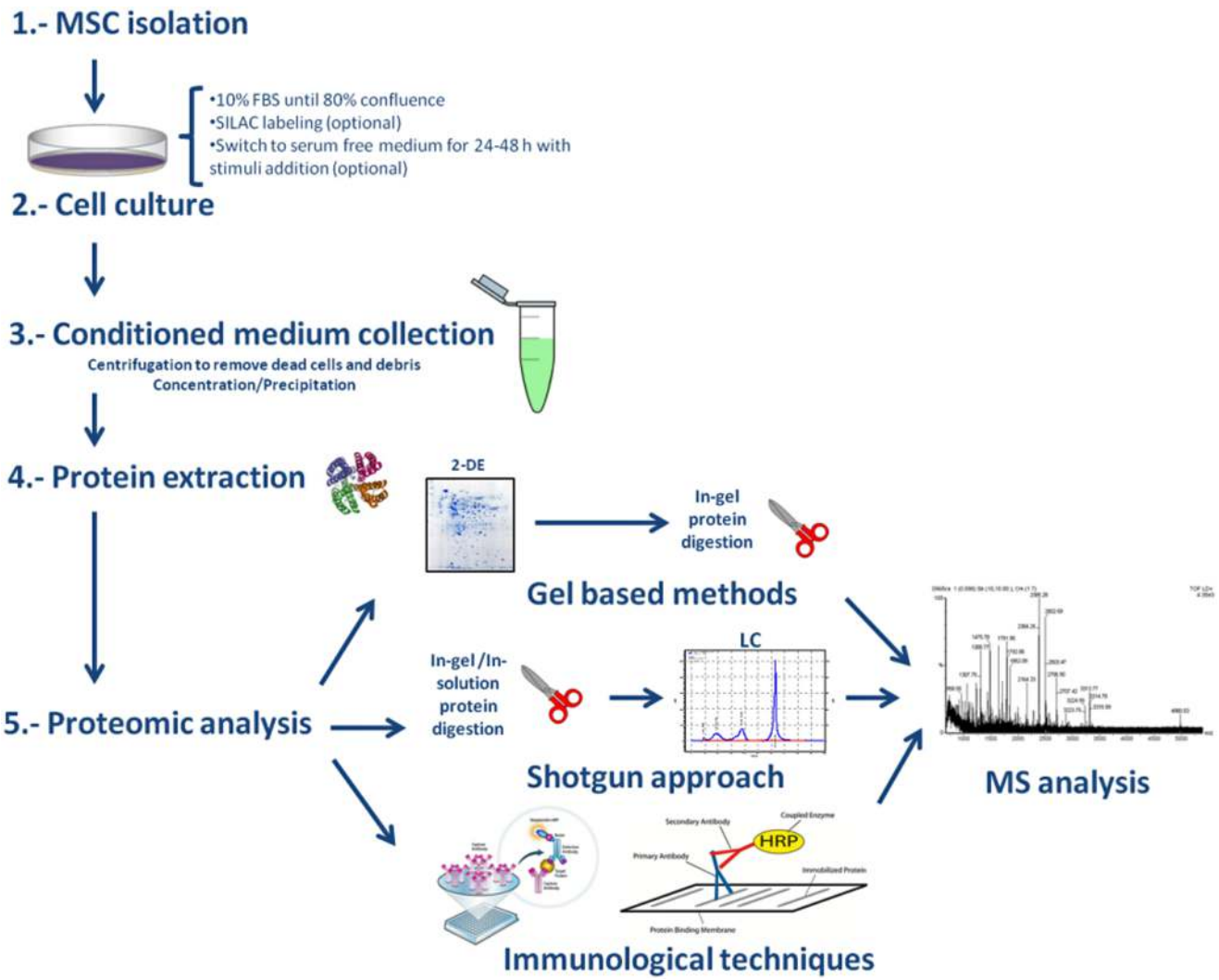



**Figure 19.1** Classical experimental workflows used in the proteomic analysis of secretomes from MSCs. MSC: mesenchymal stem/stromal cell; FBS: fetal bovine serum; 2-DE: two-dimensional electrophoresis; MS, mass spectrometry.

#### 19.2.2.2 Preparation of secretome samples

Prior to obtaining the CM the cells are extensively washed up to six times in serum-free medium or phosphate-buffered saline to remove the amount of FBS-derived proteins contaminating the medium. The cells are then cultured in a serum-free medium. The incubation time depends on the cell type, but it usually ranges between 12 and 48 h. At the end of this time the CM is collected and centrifuged in order to remove dead cells and remaining particles. These CMs can be concentrated by centrifugal filtration using specific membranes with a cut-off of 3–10 kDa, or precipitated with trichloroacetic acid, in order to obtain the proteins. The proteins are then quantified. This is particularly important if the samples are being used for a comparative analysis when it is essential to load equal amounts of total protein from each sample to be compared. Finally, the samples can be stored at −80 °C prior to further analysis.

#### 19.2.2.3 Proteomic approaches for the analysis of mesenchymal stem/stromal cell secretomes

Once secretome samples have been obtained, the next step is their analysis by proteomic techniques. Different proteomic approaches have been employed for the large-scale analysis of proteins secreted by MSCs. These include protein separation by 2-DE followed by MS, protein separation by LC followed by MS, and antibody-based assays.

##### *19.2.2.3.1 Protein separation by two-dimensional gel electrophoresis*

The 2-DE approach involves the separation of the proteins in two dimensions on a gel. In the first dimension the proteins are separated by their isoelectric point, while in the second dimension they are separated according to their molecular weight by SDS-PAGE. Following their separation by 2-DE, protein spots

can be picked from the gels and identified using MS or LC–MS after in-gel digestion. Although 2-DE provides valuable information about intact protein content in the secretome samples, several protein classes can be overlooked due to their low abundance, extreme isoelectric points, or molecular weights.

The 2-DE approach has been applied by many researchers to study proteins secreted by MSCs. Zvonic *et al.* applied this strategy followed by MS/MS to analyze the secretome of primary cultures of adipose-derived stromal cells (ADSCs) [42], identifying a number of established adipokines, such as adiponectin and plasminogen activator inhibitor 1, as well as multiple serine protease inhibitors (serpins). Moreover 2-DE has also been employed to identify and compare the secretome from rat ADSCs with or without insulin stimulation [40].

#### *19.2.2.3.2 Protein separation by liquid chromatography followed by mass spectrometry (shotgun approach)*

Shotgun approaches are currently the techniques most commonly employed for the study of MSC secretomes. This strategy starts with the digestion of proteins present in the samples and the separation of the resulting peptides by LC. Then MS/MS involving multiple steps of MS selection is used to identify the peptides. Before protein digestion samples can be fractionated by electrophoretic techniques such as 2-DE or 1D SDS-PAGE in order to reduce their complexity. Peptide separation is usually performed by multidimensional chromatographic techniques that combine strong cation exchange chromatography with reversed-phase LC.

*19.2.2.3.2.1 Proteomic studies of mesenchymal stem/stromal cells undergoing osteogenesis* LC–MS/MS technologies were applied by Choi *et al.* to study the secretome of BM-MSCs during osteogenic differentiation. They found a member of the SPARC (secreted protein acidic and rich in cysteine – also known as osteonectin) family, SPARC-related modular calcium-binding protein 1 (SMOC1), to be highly expressed in BM-MSCs undergoing osteogenesis [31]. In another study, Kim *et al.* analyzed the extracellular protein expression profile of undifferentiated human BM-MSCs as well as osteogenically induced BM-MSCs using LC–MS/MS [33]. This analysis resulted in the identification of 315 secreted proteins, of which more than 200 were differentially expressed during osteogenesis. Interestingly, calcium homeostasis-related proteins were increased in osteoblasts, while proteins related to stem cell proliferation were decreased. Furthermore, Lee *et al.* also used LC–MS/MS to study the human ADSC secretome after treatment with TNF-α [34]. Several cytokines and chemokines (such as IL-6, IL-8, C-X-C motif chemokine ligand (CXCL) 6, and monocyte chemoattractant protein 1 (MCP-1)) and several proteases (including cathepsin L, matrix metalloproteinases, protease inhibitors, and pentraxin 3 (PTX3)) were found to be more abundant.

LC–MS/MS-based quantitative strategies have also been employed in the analysis of secreted proteins. These can be performed using isotopic labeling or label-free approaches. The SILAC method is one of the most commonly employed labeling strategies for characterization of the MSC secretome. It is believed that SILAC has higher quantitative accuracy than other labeling methods because the samples to be compared can be mixed at the cellular or protein level prior to any further sample preparation, thus minimizing handling errors. In a typical SILAC experiment two populations of cells are differentially labeled by growing them in light medium, which contains normal arginine (Arg 0) and lysine (Lys 0), or in heavy medium with different isotope variants of these two amino acids (e.g., Arg 10/Lys6). A minimum of five cell doublings is needed to ensure complete incorporation of the heavy amino acids into the proteins synthesized by the cell. Proteins in the CM from the two cell populations are usually combined and separated by 1D SDS-PAGE. After in-gel digestion of the gel bands, the resulting peptide mixtures are analyzed by LC–MS/MS. Taking into account that trypsin digests the proteins at arginine and lysine residues, every tryptic peptide obtained from the heavy condition will contain at least one labeled amino acid and can therefore be discriminated by MS.

One of the major benefits of the SILAC strategy in secretome studies is that contaminating proteins from the FBS can be easily discriminated from the MSCs-derived proteins by their absence of stable isotope incorporation [46]. SILAC labeling coupled with LC–MS/MS was applied by Kristensen *et al.* to quantitatively assess the proteins secreted during osteoblast differentiation of human BM-MSCs at days 0, 1, 4, 7, and 14, covering the transition from undifferentiated to fully differentiated osteogenic cells [44]. These authors quantified 466 potentially secreted proteins, 41 of which were directly involved in osteoblast differentiation. Eight of them were increased in the early stages of osteogenic differentiation and were validated by quantitative real-time polymerase chain reaction (qRT-PCR). They were follistatin-like 1, cathepsin D, legumain, stanniocalcin 2, selectin-like osteoblast-derived protein, protein KIAA1199, plasminogen activator inhibitor 1, and PTX3, and they clearly play an important role in the formation of osteoprogenitor cells.

Molina *et al.* [47] focused on secreted proteins that were differentially expressed during the differentiation of 3T3-L1 preadipocytes to adipocytes, a model system for studying adipogenesis. They identified 882 nuclear/secreted proteins using 5-plex SILAC labeling followed by LC–MS/MS. Interestingly, THO complex 4, a context-dependent transcriptional activator in the T cell receptor alpha enhancer complex, showed its highest expression at the middle stage of adipogenesis. This protein is a heat-stable nuclear protein and functions as a molecular chaperone. It is thought to regulate dimerization, DNA binding, and transcriptional activity of basic region-leucine zipper proteins. On the other hand, SNF2-α, a chromatin remodeling protein, was downregulated in the early stages of adipogenesis and remained so at the other time points analyzed.

#### *19.2.2.3.3 Antibody-based assays*

Antibody-based assays have been also used to examine the MSC secretome in a targeted way. These approaches offer the opportunity of carrying out multiplexed, rapid, and sensitive profiling

of samples without any need to remove serum contaminants. Among the different types of immunological assays, ELISA has been the most commonly employed method for characterizing the MSC secretome as it allows a very accurate and sensitive detection of a single antigen of interest. Several studies have examined proteins secreted from MSCs employing ELISA. Kilroy *et al.* characterized the cytokine profile of primary cultures of undifferentiated and adipocyte-differentiated human ADSCs [14] and observed hepatocyte growth factor (HGF), a cytokine implicated in hematopoiesis and vasculogenesis, to be upregulated in the ADSC secretome after basic FGF treatment. In another study, Wang *et al.* evaluated the secretome of human BM-MSCs and ADSCs after preincubation with TNF-α. They found an increased secretion of vascular endothelial growth factor (VEGF), HGF, and insulin-like growth factor 1 (IGF-1) from both cell populations [15].

Recently, multiplex assays have been developed to quantify multiple antigens across samples. Multiplex techniques have several advantages over ELISAs, including reduced time and sample requirement, the ability to evaluate one antigen in the context of many others, and the capacity to detect different proteins across a broad dynamic range. Several different multiplex formats are currently available. Bead-based assays are probably the most commonly used (e.g., the Luminex technique). In this method each bead is coated with a specific capture antibody and streptavidin-labeled antibodies bind to the specific capture antibody complex, which can be then detected by chemiluminescence or fluorescence in a flow cytometer. Antibody microarrays are also commonly used. An example is the RayBio antibody array system, which involves the immobilization of the capture antibodies on the surface of membranes or glass slides. Roche *et al.* investigated the proteomic and secretory profile of adult multilineage inducible (MIAMI) cells and human MSCs isolated from bone marrow using a multiplex assay [10]. MIAMI cells secrete higher levels of VEGF, fractalkine, IL-6, IL-8, and growth-related oncogene proteins than human MSCs do. Finally, cytokine protein arrays have been employed to analyze the secretory profile of human umbilical cord-derived mesenchymal stem/stromal cells (UCMSCs) [28] and also to study the angiogenic factors produced during BM-MSC osteogenic differentiation [20].

## 19.3 Analysis of the secretome of mesenchymal stem/stromal cells undergoing chondrogenesis

MSCs can differentiate into different cell types, including adipocytes, osteoblasts, and chondrocytes. The process by which MSCs differentiate towards chondrocytes and start secreting the molecules that form the ECM of cartilage is called chondrogenesis [58]. The chondrogenic potential of MSCs, as well as their immunomodulatory function, renders them an interesting candidate for a cell-based therapy of cartilage defects [59].

Proteomic strategies have recently been applied in order to increase knowledge of the molecular mechanisms that participate in the differentiation processes of MSCs [60]. As indicated earlier, several proteomic studies have been performed to explore the secretome of MSCs undergoing osteogenesis and adipogenesis [33,47,61]. However, extracellular protein profiles of MSCs during chondrogenesis have been less often reported, and only a few studies have been carried out to characterize the secretome from human MSCs undergoing chondrogenic differentiation. Table 19.2 lists those proteins related to the ECM of articular cartilage that have been identified so far by immunological assays and LC–MS/MS techniques in the secretome of MSCs undergoing chondrogenesis.

Among these studies Lee *et al.* focused on proteins that were differentially expressed during chondrogenesis [62]. Undifferentiated MSCs and chondrocytes were analyzed using 2-DE andMS/MS analysis. Only nine proteins could be identified among the spots that were differentially expressed between the two groups. The expression of two extracellular proteins, collagen type II, a biomarker of chondrocytes, and matrilin-1, a noncollagenous fibrillar cartilage matrix protein, was increased during chondrogenesis. Arufe *et al.* [38] used LC coupled to matrix-assisted laser desorption ionization (MALDI) with MS/MS to characterize the secretome of MSCs obtained from umbilical cord at different time points during chondrogenesis. In MALDI imaging MS, the sample, often a thin tissue section, is moved in two dimensions while the mass spectrum is recorded. Advantages such as measuring the distribution of a large amount of analytes at one time without destroying the sample make it a useful method in tissue-based studies.

Using this strategy, Arufe *et al.* identified 209 proteins that were directly linked to extracellular processes during chondrogenesis. These included ECM-related proteins such as basement-membrane-specific heparan sulfate proteoglycan core protein and several collagens, several cartilage proteases, such as 72 kDa type IV collagenase (MMP2) and interstitial collagenase (MMP1) and their tissue inhibitors metalloproteinase inhibitor (TIMP) 1 and TIMP2. Other proteins involved in cell adhesion, such as fibronectin or dermatopontin, were also identified. These findings and those referenced in Section 19.4 have increased the knowledge on the molecular mechanisms occurring during chondrogenesis. The identification of several proteins related to cell growth and differentiation in the secretomes of MSCs during induction of chondrogenesis (Table 19.3) is valuable for the monitoring of this process *in vitro*.

## 19.4 Characterization of chondrogenesis markers by secretome analysis

One of the applications for which the study of MSC secretomes has major advantages is the identification of differentiation markers that could be applied in cell therapy-based approaches.

**Table 19.2** Known cartilage ECM-related proteins identified in the secretome of MSCs undergoing chondrogenesis by immunological and LC–MS/MS techniques.

| Protein name | Reference |
|---|---|
| Annexin A2 | [38,45] |
| Apolipoprotein D and E | [45] |
| Apolipoprotein E | [45] |
| Basement-membrane-specific heparan sulfate proteoglycan core protein | [38,45] |
| Biglycan | [36,38,45] |
| Cartilage acidic protein 1 | [45] |
| Cartilage oligomeric matrix protein | [45] |
| CD59 glycoprotein | [45] |
| Chondroitin sulfate proteoglycan 4 | [45] |
| Collagen α-1 and α-2 (I) chains | [38,45] |
| Collagen α-1 (III) chain | [38] |
| Collagen α-1 and α-2 (IV) chains | [45] |
| Collagen α-1 and α-3 (V) chains | [45] |
| Collagen α-1 and α-3 (V) chains | [45] |
| Collagen α-1, α-2 and α-3 (V) chains | [38,45] |
| Collagen α-1 (VIII, XI, XII, XIV, XV, XVI, XVIII) chains | [45] |
| Collagen triple helix repeat-containing protein 1 | [45] |
| Decorin | [36,38,45] |
| Dystroglycan | [45] |
| EGF-containing fibulin-like ECM proteins 1 and 2 | [45] |
| Fibrillin-1 | [45] |
| Fibromodulin | [45] |
| Fibulin-1 and 2 | [38,45] |
| Glypican-1 and 6 | [45] |
| Latent-transforming growth factor beta-binding proteins 1, 2 and 3 | [45] |
| Lumican | [36,38,45] |
| Lysyl oxidase homologs 1, 2, and 3 | [45] |
| Microfibrillar-associated protein 5 | [45] |
| Peroxidasin homolog | [45] |
| Prolargin | [45] |
| Proteoglycan 4 | [45] |
| Stanniocalcin-1 and -2 | [45] |
| Syndecan-4 | [45] |
| TNF receptor superfamily member 11B | [45] |

Quantitative proteomic analyses have been performed to characterize the protein modulations in the secretome of BM-MSCs during the first steps of their differentiation towards chondrocyte-like cells. One of these studies was carried out by our group using a double-SILAC strategy coupled to LC–MS/MS. The workflow of this procedure is shown in Figure 19.2.

The double-SILAC method avoids quantification artifacts from the FBS employed in the cell culture. Two populations of human BM-MSCs are grown with isotope variants of lysine (Lys) and arginine (Arg) (Arg6, Lys4 for the "medium" labeling and Arg10, Lys8 for the "heavy" labeling) for 4–6 weeks until full labeling had been achieved. The labeled populations are then

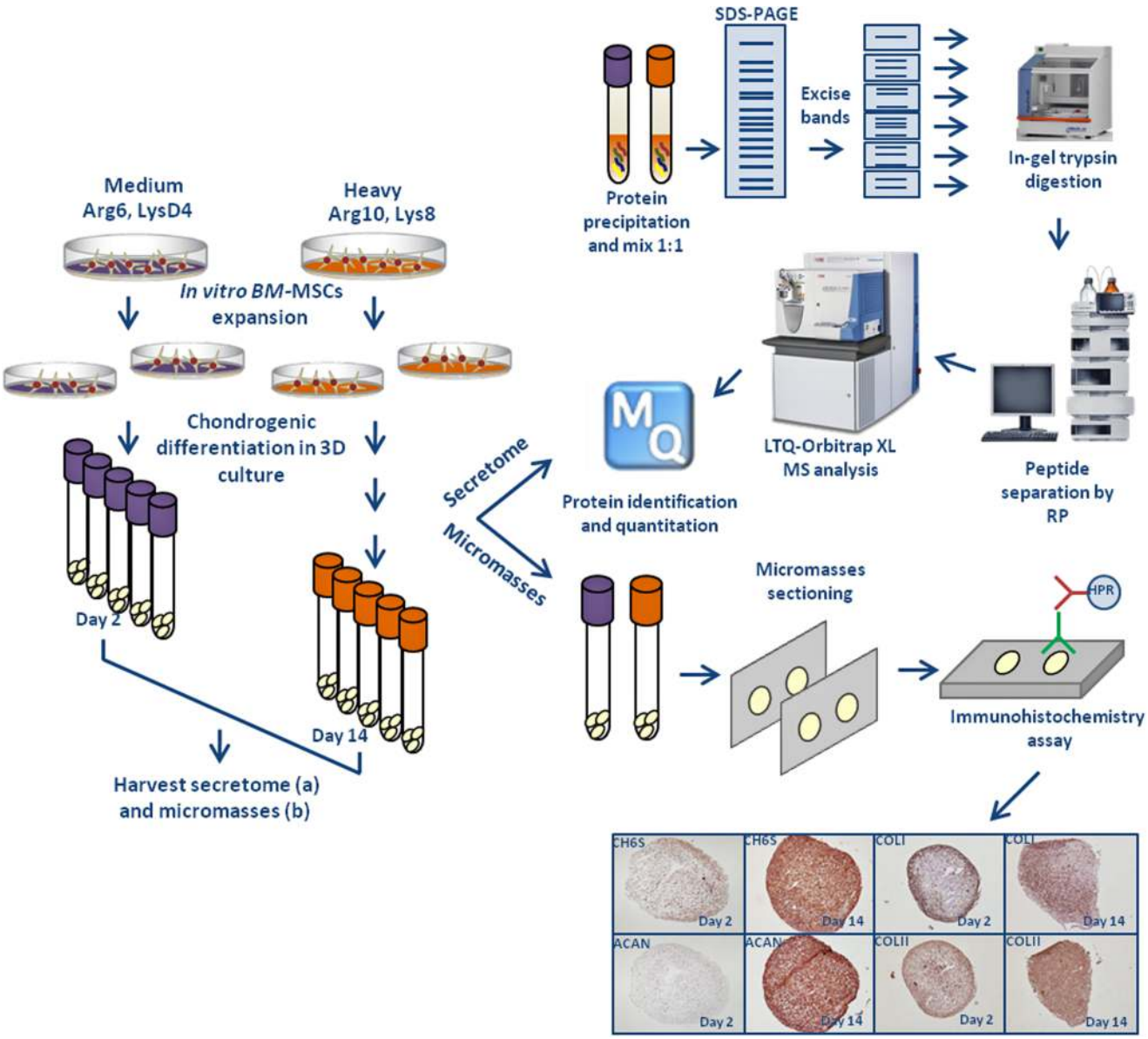



**Figure 19.2** Experimental workflow for the secretome analysis of BM-MSCs undergoing chondrogenesis. BM-MSCs, bone marrow mesenchymal stem/stromal cells; RP, reversed phase; HPR, horseradish peroxidase; COL2, type II collagen; COL1, type I collagen; ACAN, aggrecan; CH6S, chondroitin-6-sulfate. (See color plate section.)

subjected to differentiation in three-dimensional cultures (micromasses) according to standard procedures [63]. For stimulation of chondrogenesis a medium with TGF-β3 among other chondrogenic inducers [64] can be employed. At different days of differentiation (in our case 2 and 14 days) the micromasses are washed in serum-free media and incubated overnight with serum-free labeling medium supplemented with chondrogenic inducers. CM is then collected after 24 h, and the micromass pellets are also collected to perform verification studies of chondrogenesis, including immunohistochemical analysis of collagens (types I and II) and proteoglycans (aggrecan and chondroitin 6 sulfate). For the proteomic analysis, proteins in the CM are precipitated and protein samples from the two different time points to be compared are mixed 1 : 1 and separated into eight fractions by SDS-PAGE. After in-gel digestion of each fraction, the resulting peptide mixtures can be analyzed by LC coupled to MS. In our case we used an LC-linear trap quadripole Orbitrap XL instrument and quantified the proteins using MaxQuant software. Validation of the putative biomarker candidates of chondrogenesis can be carried out using orthogonal techniques such as Western blot or immunohistochemistry assays.

This study led to the identification of 221 proteins with a reported extracellular localization, many of which are

summarized in Tables 19.2 and 19.3. Most of them were characteristic of the ECM of articular cartilage, and 34 showed statistically significant quantitative modulations during chondrogenesis when comparing the secretome protein profiles between days 2 and 14 of differentiation. These proteins are listed in Table 19.4 and include typical cartilage markers, such as proteoglycan 4 (PRG4/lubricin) or cartilage oligomeric matrix protein (COMP), anticatabolic markers such as TIMP1, reported markers of cartilage development (versican), and a suggested marker of chondrogenesis, cartilage acidic protein 1 (CRAC1). All of them were increased at day 14 of chondrogenesis. Conversely, the only four proteins detected with a significant reduction in their amounts at day 14 compared with day 2 were PTX3, tenascin (TENA), Thy-1 membrane glycoprotein (THY1, CD90), and chondroitin sulfate proteoglycan 4 (CSPG4).

Apart from this work, Bara *et al.* have used an LC–MS/MS approach to profile the CM obtained from equine BM-MSCs [36]: multiple fibronectin peptides as well as peptides

**Table 19.3** Proteins involved in cell growth and differentiation identified by immunological and LC–MS/MS techniques in the secretome of MSCs undergoing chondrogenesis.

| Protein name | References |
|---|---|
| Angiogenin | [45] |
| Angiopoietin 2 | [36] |
| Angiopoietin-related proteins 2 and 4 | [45] |
| Bone morphogenetic protein 6 | [45] |
| C-type lectin domain family 11 member A | [45] |
| Endothelin-1 | [36] |
| Extracellular matrix protein 1 | [45] |
| Extracellular serine/threonine protein kinase Fam20C | [45] |
| Fibroblast growth factors 2, 4, and 7 | [36] |
| Follistatin | [45] |
| Follistatin-related proteins 1 and 3 | [38,45] |
| Glial cell line-derived neurotrophic factor | [36] |
| Galectin-1 and -3 | [38,45] |
| Gremlin-1 | [45] |
| Granulins | [36] |
| Inhibin beta A chain | [45] |
| Insulin-like growth factor-binding proteins 1, 2, 3, 4, 5, 6, and 7 | [36,38,45] |
| Insulin-like growth factor II | [45] |
| Leukemia inhibitory factor | [45] |
| Matrix Gla protein | [45] |
| Neuropilin-1 | [45] |
| Protein CREG1 | [45] |
| Protein-lysine 6-oxidase | [45] |
| Secreted frizzled-related proteins 1 and 4 | [45] |
| Semaphorin-3C | [45] |
| Serglycin | [45] |
| SPARC | [38,45] |
| SPARC-related modular calcium-binding protein 1 | [45] |
| Tetranectin | [45] |
| Transforming growth factor beta-1 | [45] |
| Twisted gastrulation protein homolog 1 | [45] |
| Vascular endothelial growth factor A | [36,45] |

**Table 19.4** Extracellular proteins modulated during the early steps of chondrogenesis that were identified in human BM-MSCs secretomes.

| Acc. no. | Entry | Protein name | Modulation (day 14 vs day 2) |
|---|---|---|---|
| Q9NQ79 | CRAC1 | Cartilage acidic protein 1 | Increased |
| P49747 | COMP | Cartilage oligomeric matrix protein | Increased |
| Q6UVK1 | CSPG4 | Chondroitin sulfate proteoglycan 4 | Decreased |
| P10909 | CLUS | Clusterin | Increased |
| P08123 | CO1A2 | Collagen alpha-2(I) chain | Increased |
| P01034 | CYTC | Cystatin-C | Increased |
| O75462 | CRLF1 | Cytokine receptor-like factor 1 | Increased |
| Q8IXL6 | DMP4 | Extracellular serine/threonine protein kinase Fam20C | Increased |
| P02794 | FRIH | Ferritin heavy chain | Increased |
| Q06828 | FMOD | Fibromodulin | Increased |
| Q12841 | FSTL1 | Follistatin-related protein 1 | Increased |
| O14498 | ISLR | Immunoglobulin superfamily containing leucine-rich repeat protein | Increased |
| P22692 | IBP4 | Insulin-like growth factor-binding protein 4 | Increased |
| P24592 | IBP6 | Insulin-like growth factor-binding protein 6 | Increased |
| Q16270 | IBP7 | Insulin-like growth factor-binding protein 7 | Increased |
| Q14766 | LTBP1 | Latent-transforming growth factor beta-binding protein | Increased |
| P51884 | LUM | Lumican | Increased |
| P08493 | MGP | Matrix Gla protein | Increased |
| P01033 | TIMP | Metalloproteinase inhibitor 1 | Increased |
| P16035 | TIMP2 | Metalloproteinase inhibitor 2 | Increased |
| P29120 | NEC1 | Neuroendocrine convertase 1 | Increased |
| P43490 | NAMPT | Nicotinamide phosphoribosyltransferase | Increased |
| P26022 | PTX3 | Pentraxin-related protein PTX3 | Decreased |
| Q15113 | PCOC1 | Procollagen C-endopeptidase enhancer 1 | Increased |
| P51888 | PRELP | Prolargin | Increased |
| Q92954 | PRG4 | Proteoglycan 4 | Increased |
| Q92743 | HTRA1 | Serine protease HTRA1 | Increased |
| P52823 | STC1 | Stanniocalcin-1 | Increased |
| O76061 | STC2 | Stanniocalcin-2 | Increased |
| P24821 | TENA | Tenascin | Decreased |
| P04216 | THY1 | Thy-1 membrane glycoprotein | Decreased |
| P10646 | TFPI1 | Tissue factor pathway inhibitor | Increased |
| P48307 | TFPI2 | Tissue factor pathway inhibitor 2 | Increased |
| P13611 | CSPG2 | Versican core protein | Increased |

of cartilage-associated matrix proteins, including biglycan and decorin, were identified in the chondrogenic CM. Type II collagen and lumican were also detected in two of the three samples analyzed. In addition, angiogenesis- and neurogenesis-related factors were screened using protein arrays against 55 proteins, leading to the detection of five angiogenic factors (VEGF, FGF-4, FGF-7, MCP-1, and activin A) at high levels during the early stages of chondrogenesis. Other work has focused specifically on the subset of glycosylated proteins in the secretome of MSCs [65]. Glycosylation is one of the most important posttranslational modifications of cell surface proteins and extracellular proteins. For this reason, glycoprotein enrichment techniques can be applied to increase the concentration of glycoproteins and eliminate cytoplasmic proteins. Ishihara *et al.* employed a

proteomics platform based on glycoprotein enrichment coupled to LC–MS to discover novel chondrogenic differentiation markers. From the total of 246 *N*-glycoproteins identified, 53 were either localized on the ECM or secreted. Among them, two well-known cartilage collagens, collagen alpha-1 (III) chain and collagen alpha-1(XI) chain, were upregulated during chondrogenic differentiation. In addition, increased expression of fibronectin and granulins was also found after 18 days of differentiation.

## 19.5 Characterization of osteogenesis markers by secretome analysis

As mentioned earlier in this chapter, Choi *et al.* studied the secretome of BM-MSCs during osteogenic differentiation and found a member of the SPARC family, SMOC1, to be highly expressed in BM-MSCs undergoing osteogenesis [31]. In another study, Kim *et al.* analyzed the extracellular protein expression profile of undifferentiated human BM-MSCs as well as osteogenically induced BM-MSCs using LC–MS/MS [33]. This analysis resulted in the identification of 315 secreted proteins, of which more than 200 were differentially expressed during osteogenesis. Interestingly, calcium homeostasis-related proteins were increased in osteoblasts, while proteins related to stem cell proliferation were decreased.

SILAC labeling coupled with LC–MS/MS was applied by Kristensen *et al.* to quantitatively assess the proteins secreted during osteoblast differentiation of human BM-MSCs at days 0, 1, 4, 7, and 14, covering the transition from undifferentiated to fully differentiated osteogenic cells [44]. They quantified 466 potentially secreted proteins, 41 of which were directly involved in osteoblast differentiation. Eight of them were increased in the early stages of osteogenic differentiation and were validated by qRT-PCR. They were follistatin-like 1, cathepsin D, legumain, stanniocalcin 2, selectin-like osteoblast-derived protein, protein KIAA1199, plasminogen activator inhibitor 1, and PTX3, and they clearly play an important role in the formation of osteoprogenitor cells.

## 19.6 Characterization of adipogenesis markers by secretome analysis

Again as mentioned earlier, Zvonic *et al.* analyzed the secretome of primary cultures of adipose-derived stromal cells (ADSCs) [42] and identified a number of established adipokines, such as adiponectin and plasminogen activator inhibitor 1, as well as multiple serine protease inhibitors (serpins). Lee *et al.* also used LC–MS/MS to study the human ADSC secretome after treatment with TNF-α [34]. Several cytokines and chemokines (such as IL-6, IL-8, CXCL6, and MCP-1) and several proteases (including cathepsin L, matrix metalloproteinases, protease inhibitors, and PTX3) were found to be more abundant in the ADSCs preincubated with TNF-α.

Molina *et al.* [47] focused on secreted proteins that were differentially expressed during the differentiation of 3T3-L1 preadipocytes to adipocytes, a model system for studying adipogenesis. They identified 882 nuclear/secreted proteins using five-plex SILAC labeling followed by LC–MS/MS. Interestingly, THO complex 4, a context-dependent transcriptional activator in the T cell receptor alpha enhancer complex, showed its highest expression at the middle stage of adipogenesis.

Kilroy *et al.* characterized the cytokine profile of primary cultures of undifferentiated and adipocyte-differentiated human ADSCs [14] and observed HGF, a cytokine implicated in hematopoiesis and vasculogenesis, to be upregulated in the ADSC secretome after basic FGF treatment. In another study, Wang *et al.* evaluated the secretome of human BM-MSCs and ADSCs after preincubation with TNF-α. They found an increased secretion of VEGF, HGF, and IGF-I from both cell populations [15].

## 19.7 Conclusions and future perspectives

This chapter summarizes studies performed to increase knowledge on the molecular mechanisms that drive chondrogenic differentiation of MSCs, and to a lesser extent on those that drive MSC differentiation to osteogenesis and those that drive MSC differentiation to adipogenesis.

The referenced studies demonstrate the usefulness of secretome analysis for understanding the mechanisms responsible for cartilage matrix formation and have described panels of extracellular/secreted markers potentially useful for the evaluation of tissue development in cell therapy- or tissue-engineering-based approaches for cartilage repair. Further efforts should be made to improve the applicability of these strategies to clinical practice. In osteoarthritis, compounds with chondrogenic capacity have recently emerged as a good option for the development of new disease-modifying drugs [66]. The existence of validated chondrogenesis markers will be useful for all such initiatives.

## References

1 H. Tjalsma, A. Bolhuis, J.D. Jongbloed, *et al.* Signal peptide-dependent protein transport in *Bacillus subtilis*: a genome-based survey of the secretome. *Microbiol Mol Biol Rev* 2000;**64**:515–547.
2 M.P. Pavlou and E. P. Diamandis. The cancer cell secretome: a good source for discovering biomarkers? *J Proteomics* 2010;**73**:1896–1906.
3 M. Makridakis and A. Vlahou. Secretome proteomics for discovery of cancer biomarkers. *J Proteomics* 2010;**73**:2291–2305.
4 S.A. Mustafa, J.D. Hoheisel, and M.S. Alhamdani. Secretome profiling with antibody microarrays. *Mol Biosyst* 2011;**7**:1795–1801.
5 H. Skalnikova, J. Motlik, S.J. Gadher, and H. Kovarova. Mapping of the secretome of primary isolates of mammalian cells, stem cells and derived cell lines. *Proteomics* 2011;**11**:691–708.

6 Y. Hathout. Approaches to the study of the cell secretome. *Expert Rev Proteomics* 2007;**4**:239–248.

7 W. Nickel and C. Rabouille. Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* 2009;**10**:148–155.

8 A. Bhattacharya, Y.S. Prakash, and N.T. Eissa. Secretory function of autophagy in innate immune cells. *Cell Microbiol* 2014;**16**: 1637–1645.

9 W. Nickel. Unconventional secretory routes: direct protein export across the plasma membrane of mammalian cells. *Traffic* 2005;**6**:607–614.

10 S. Roche, G. D'Ippolito, L.A. Gomez, *et al.* Comparative analysis of protein expression of three stem cell populations: models of cytokine delivery system *in vivo*. *Int J Pharm* 2013;**440**:72–82.

11 C. Nakanishi, N. Nagaya, S. Ohnishi, *et al. Gene and protein expression analysis of mesenchymal stem cells derived from rat adipose tissue and bone marrow. Circ J* 2011;**75**:2260–2268.

12 H. Li, S. Zuo, Z. He, *et al.* Paracrine factors released by GATA-4 overexpressed mesenchymal stem cells increase angiogenesis and cell survival. *Am J Physiol Heart Circ Physiol* 2010;**299**: H1772–H1781.

13 X. Qu, X. Liu, K. Cheng, *et al.* Mesenchymal stem cells inhibit Th17 cell differentiation by IL-10 secretion. *Exp Hematol* 2012;**40**: 761–770.

14 G.E. Kilroy, S.J. Foster, X. Wu, *et al.* Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J Cell Physiol* 2007;**212**:702–709.

15 M. Wang, P.R. Crisostomo, C. Herring, *et al.* Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol* 2006;**291**:R880–R884.

16 J. Rehman, D. Traktuev, J. Li, *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;**109**:1292–1298.

17 P.M. Campeau, M. Rafei, M.N. Boivin, *et al.* Characterization of Gaucher disease bone marrow mesenchymal stromal cells reveals an altered inflammatory secretome. *Blood* 2009;**114**:3181–3190.

18 G. Ren, L. Zhang, X. Zhao, *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**:141–150.

19 C.A. Ribeiro, J.S. Fraga, M. Grãos, *et al.* The secretome of stem cells isolated from the adipose tissue and Wharton jelly acts differently on central nervous system derived cell populations. *Stem Cell Res Ther* 2012;**3** (3): *18*

20 A.I. Hoch, B.Y. Binder, D.C. Genetos, and J.K. Leach. Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PLoS ONE* 2012;**7**:e35579.

21 A. Oskowitz, H. McFerrin, M. Gutschow, *et al.* Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. *Stem Cell Res* 2011;**6**:215–225.

22 J.A. Potian, H. Aviv, N.M. Ponzio, *et al.* Veto-like activity of mesenchymal stem cells: functional discrimination between cellular responses to alloantigens and recall antigens. *J Immunol* 2003; **171**:3426–3434.

23 S. Grässel, N. Ahmed, C. Göttl, and J. Grifka. Gene and protein expression profile of naive and osteo-chondrogenically differentiated rat bone marrow-derived mesenchymal progenitor cells. *Int J Mol Med* 2009;**23**:745–755.

24 M. Rafei, J. Hsieh, S. Fortier, *et al.* Mesenchymal stromal cell-derived CCL2 suppresses plasma cell immunoglobulin production via STAT3 inactivation and PAX5 induction. *Blood* 2008;**112**: 4991–4998.

25 H. Zhang, R. Yang, Z. Wang, *et al.* Adipose tissue-derived stem cells secrete CXCL5 cytokine with neurotrophic effects on cavernous nerve regeneration. *J Sex Med* 2011;**8**:437–446.

26 E. Maury, K. Ehala-Aleksejev, Y. Guiot, *et al.* Adipokines oversecreted by omental adipose tissue in human obesity. *Am J Physiol Endocrinol Metab* 2007;**293**:E656–E665.

27 S.K. Sze, D.P. de Kleijn, R.C. Lai, *et al.* Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteomics* 2007;**6**:1680–1689.

28 C.H. Liu and S.M. Hwang. Cytokine interactions in mesenchymal stem cells from cord blood. *Cytokine* 2005;**32**:270–279.

29 H. Sarojini, R. Estrada, H. Lu, *et al.* PEDF from mouse mesenchymal stem cell secretome attracts fibroblasts. *J Cell Biochem* 2008;**104**: 1793–1802.

30 R. Estrada, N. Li, H. Sarojini, *et al.* Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. *J Cell Physiol* 2009;**219**: 563–571.

31 Y.A. Choi, J. Lim, K.M. Kim, *et al.* Secretome analysis of human BMSCs and identification of SMOC1 as an important ECM protein in osteoblast differentiation. *J Proteome Res* 2010;**9**: 2946–2956.

32 H.S. Kim, D.Y. Choi, S.J. Yun, *et al.* Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J Proteome Res* 2012;**11**:839–849.

33 J.M. Kim, J. Kim, Y.H. Kim, *et al.* Comparative secretome analysis of human bone marrow-derived mesenchymal stem cells during osteogenesis. *J Cell Physiol* 2013;**228**:216–224.

34 M.J. Lee, J. Kim, M.Y. Kim, *et al.* Proteomic analysis of tumor necrosis factor-alpha-induced secretome of human adipose tissue-derived mesenchymal stem cells. *J Proteome Res* 2010;**9**:1754–1762.

35 K.M. Akram, S. Samad, M.A. Spiteri, and N.R. Forsyth. Mesenchymal stem cells promote alveolar epithelial cell wound repair *in vitro* through distinct migratory and paracrine mechanisms. *Respir Res* 2013;**14**:9.

36 J.J. Bara, H.E. McCarthy, E. Humphrey, *et al.* Bone marrow-derived mesenchymal stem cells become antiangiogenic when chondrogenically or osteogenically differentiated: implications for bone and cartilage tissue engineering. *Tissue Eng Part A* 2014;**20**:147–159.

37 C. Chiellini, O. Cochet, L. Negroni, *et al.* Characterization of human mesenchymal stem cell secretome at early steps of adipocyte and osteoblast differentiation. *BMC Mol Biol* 2008;**9**:26.

38 M.C. Arufe, A. De la Fuente, J. Mateos, *et al.* Analysis of the chondrogenic potential and secretome of mesenchymal stem cells derived from human umbilical cord stroma. *Stem Cells Dev* 2011;**20**:1199–1212.

39 D. Purmessur, R.M. Schek, R.D. Abbott, *et al.* Notochordal conditioned media from tissue increases proteoglycan accumulation and promotes a healthy nucleus pulposus phenotype in human mesenchymal stem cells. *Arthritis Res Ther* 2011;**13**:R81.

40 X. Chen, S.W. Cushman, L.K. Pannell, and S. Hess. Quantitative proteomic analysis of the secretory proteins from rat adipose cells using a 2D liquid chromatography–MS/MS approach. *J Proteome Res* 2005;**4**:570–577.

41 D.L. Coutu, J.H. Wu, A. Monette, *et al.* Periostin, a member of a novel family of vitamin K-dependent proteins, is expressed by mesenchymal stromal cells. *J Biol Chem* 2008;**283**:17991–18001.

42 S. Zvonic, M. Lefevre, G. Kilroy, *et al.* Secretome of primary cultures of human adipose-derived stem cells: modulation of serpins by adipogenesis. *Mol Cell Proteomics* 2007;**6**:18–28.

43 R. Tasso, M. Gaetani, E. Molino, *et al.* The role of bFGF on the ability of MSC to activate endogenous regenerative mechanisms in an ectopic bone formation model. *Biomaterials* 2012;**33**:2086–2096.

44 L.P. Kristensen, L. Chen, M.O. Nielsen, *et al.* Temporal profiling and pulsed SILAC labeling identify novel secreted proteins during *ex vivo* osteoblast differentiation of human stromal stem cells. *Mol Cell Proteomics* 2012;**11**:989–1007.

45 B. Rocha, V. Calamia, V. Casas, *et al.* Secretome analysis of human mesenchymal stem cells undergoing chondrogenic differentiation. *J Proteome Res* 2014;**13**:1045–1054.

46 G. Alvarez-Llamas, E. Szalowska, M.P. de Vries, *et al.* Characterization of the human visceral adipose tissue secretome. *Mol Cell Proteomics* 2007;**6**:589–600.

47 H. Molina, Y. Yang, T. Ruch, *et al.* Temporal profiling of the adipocyte proteome during differentiation using a five-plex SILAC based strategy. *J Proteome Res* 2009;**8**:48–58.

48 K.J. Brown, C.A. Formolo, H. Seol, *et al.* Advances in the proteomic investigation of the cell secretome. *Expert Rev Proteomics* 2012; **9**:337–345.

49 M. Polacek, J.A. Bruun, O. Johansen, and I. Martinez. Differences in the secretome of cartilage explants and cultured chondrocytes unveiled by SILAC technology. *J Orthop Res* 2010;**28**:1040–1049.

50 L. Lourido, V. Calamia, J. Mateos, *et al.* Quantitative proteomic profiling of human articular cartilage degradation in osteoarthritis. *J Proteome Res* 2014;**13** (12):6096–6106.

51 S. Gronthos, A.C. Zannettino, S.J. Hay, *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003;**116**:1827–1835.

52 P. Pires de Carvalho, K.M. Hamel, R. Duarte, *et al.* Comparison of infrapatellar and subcutaneous adipose tissue stromal vascular fraction and stromal/stem cells in osteoarthritic subjects. *J Tissue Eng Regen Med* 2014;**8**:757–762.

53 R.J. Jankowski, B.M. Deasy, and J. Huard. Muscle-derived stem cells. *Gene Ther* 2002;**9**:642–647.

54 R. Tuli, S. Tuli, S. Nandi, and R.S. Tuan. Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* 2003;**21**:681–693.

55 M.Q. Wickham, G.R. Erickson, J.M. Gimble, *et al.* Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clin Orthop Relat Res* 2003;**412**:196–212.

56 B. Johnstone, T.M. Hering, A.I. Caplan, *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**:265–272.

57 A.M. Mackay, S.C. Beck, J.M. Murphy, *et al.* Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* 1998;**4**:415–428.

58 M.B. Goldring, K. Tsuchimochi, and K. Ijiri. The control of chondrogenesis. *J Cell Biochem* 2006;**97**:33–44.

59 P.K. Gupta, A.K. Das, A. Chullikana, and A.S. Majumdar. Mesenchymal stem cells for cartilage repair in osteoarthritis. *Stem Cell Res Ther* 2012;**3** (4): *25*

60 M. Maurer. Proteomic definitions of mesenchymal stem cells. *Stem Cells Int* 2011;**2011**:704256.

61 J. Zhong, S.A. Krawczyk, R. Chaerkady, *et al.* Temporal profiling of the secretome during adipogenesis in humans. *J Proteome Res* 2010;**9**:5228–5238.

62 S.J. Lee, H.B. Jeon, J.H. Lee, *et al.* Identification of proteins differentially expressed during chondrogenesis of mesenchymal cells. *FEBS Lett* 2004;**563**:35–40.

63 C. Cicione, S. Díaz-Prado, E. Muiños-López, *et al.* Molecular profile and cellular characterization of human bone marrow mesenchymal stem cells: donor influence on chondrogenesis. *Differentiation* 2010;**80**:155–165.

64 S. Boeuf and W. Richter. Chondrogenesis of mesenchymal stem cells: role of tissue source and inducing factors. *Stem Cell Res Ther* 2010;**1**:31.

65 T. Ishihara, K. Kakiya, K. Takahashi, *et al.* Discovery of novel differentiation markers in the early stage of chondrogenesis by glycoform-focused reverse proteomics and genomics. *Biochim Biophys Acta* 2014;**1840**:645–655.

66 F.J. Blanco and C. Ruiz-Romero. New targets for disease modifying osteoarthritis drugs: chondrogenesis and Runx1. *Ann Rheum Dis* 2013;**72**:631–634.

CHAPTER 20

# Mesenchymal stromal cell extracellular vesicles/exosomes

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## 20.1 From cell to secretion to exosome

### 20.1.1 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are presently the most prominent stromal cell candidate in the field of regenerative medicine. They are being tested against an increasingly wide spectrum of disease indications, as reflected in the threefold increase in MSC-based product investigational new drug (IND) submissions to the USA Food and Drug Administration (FDA) between 2006 and 2012 [1]. Four hundred trials using MSCs were listed in the ClinicalTrials.gov database hosted by the USA National Institutes of Health (http://www.clinicaltrials.gov/, as of September 2014) [2]. MSCs are multipotent stromal cells. While it is generally reported that their differentiation potential is limited to osteocytes, chondrocytes, adipocytes, and myoblasts [3], they have also been reported to differentiate into other cell types outside the mesenchymal lineage, although very few, if any, of such cells have been shown to have functional activity [4]. This potential to differentiate and replace cells in injured tissues together with the relative ease of isolating and expanding these cells *ex vivo* from ethically acceptable adult tissues has provided a compelling rationale for the use of MSCs in clinical trials [2,5–7].

With the intense clinical evaluation of MSCs as potential therapeutic agents, the initial differentiation-based rationale for their use came under heavy scrutiny and stringent evaluation. Many studies reporting functional improvement after MSC transplantation observed that MSCs' engraftment and their subsequent differentiation into appropriate cell types were rare [8–10]. Although migration, engraftment, and differentiation of MSCs at sites of injury to restore tissue function after transplantation in animal models have been widely reported, the number of cells actually reaching the target tissue is <1% of those transplanted, with most of the cells being trapped in the liver, spleen, and lung [11]. Furthermore, reported evidence for differentiation of transplanted MSCs at the site of injury was often limited to the presence of new phenotypic traits in the transplanted cells, which could be acquired through fusion with endogenous host cells [8–10]. The therapeutic efficacy of transplanted MSCs was noted to be independent of their physical

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proximity to the tissue [12–15]. Together, these observations contradict the cell differentiation and replacement hypothesis and led to the proposal that MSCs mediate their effects through their secretions [16].

### 20.1.2 Cell secretion

Haynesworth *et al.* first described the paracrine effect of MSCs two decades ago when they reported that MSCs synthesize and secrete a broad spectrum of growth factors, chemokines, and cytokines that could exert significant effects on cells in their vicinity [17]. This report was followed by numerous other reports that these secreted factors enhance arteriogenesis [18], protect against ischemic renal and limb tissue injury [19], promote neovascularization [20], and increase angiogenesis [21,22]. Gnecchi *et al.* demonstrated that intramyocardial injection of culture medium conditioned by MSCs overexpressing the *Akt* gene reduced infarct size in a rodent model of acute myocardial infarction to the same extent as the cells themselves [23]. Our group subsequently demonstrated that human MSCs derived from embryonic stem cells secrete more than 200 unique gene products [24] into their culture medium and this conditioned culture medium could reduce myocardial ischemia/reperfusion injury in a pig model [25]. Together, these studies provided pivotal support for the paracrine hypothesis such that MSC therapy is increasingly rationalized on MSC secretions rather than their differentiation potential [23,26–34].

Many of the initial efforts to identify the active therapeutic agent in MSC secretion centered on small molecules such as growth factors, chemokines,and cytokines [16]. Despite many promising candidate molecules, none could sufficiently account for the efficacy of MSCs (reviewed in [35]). For example, the immunotherapeutic efficacy of MSCs [36] was reported to be mediated by small molecules such as interferon-γ (IFN-γ) [37–39], a downstream IFNγ-inducible gene indoleamine 2,3-dioxygenase (IDO) [40], and inducible nitric oxide synthase (iNOS) [41]. However, none of these factors survived subsequent scientific scrutiny. IFN-γ and IDO were eliminated as candidates when the lack of IDO production caused by either defective IFN-γ receptor 1 or IDO inhibitors failed to ameliorate the immunomodulatory activity of MSCs [42]. Additionally, murine MSCs, which do not have IDO activity, exert immunomodulatory effects similar to those of human MSCs, which do have IDO activity [43,44]. iNOS was similarly eliminated as a candidate as it was expressed in mouse but not human MSCs [36]. MSCs are also known to secrete many generic immunomodulating factors, such as transforming growth factor-β1, hepatocyte growth factor, heme oxygenase 1, interleukin (IL)-6, prostaglandin $E_2$, and human leukocyte antigen-G5 [43,45–55], and it is unlikely that any one of these factors could sufficiently mediate the immunotherapeutic efficacy of MSCs.

### 20.1.3 Mesenchymal stromal cell extracellular vesicles as the active therapeutic factor

The failure to identify the efficacious molecule in MSC secretion in the complex diseases being evaluated in MSC clinical trials is compelling evidence that the secreted agent is likely to be equally complex. As early as 2008 we observed that 0.2 μm filtered culture medium conditioned by MSCs was cardioprotective [25,33]. However, when this medium was filtered through 1000 kDa filter, the >1000 kDa retentate, but not the <1000 kDa filtrate, was cardioprotective [25], placing the protective agent as a large 100–200 nm complex. In 2009 Bruno *et al.* described the efficacy of a population of MSC extracellular vesicles (EVs), which they termed microvesicles and which ameliorated glycerol-induced acute kidney injury [56]. These microvesicles had an estimated diameter of 180 nm. The authors attributed the renal protection of these EVs to their cargo of MSC RNAs, since RNase pretreatment of the EVs abrogated the protective effect. In contrast, our group, using size-exclusion high-performance liquid chromatography, reported that the therapeutic agent had a hydrodynamic radius of 55–65 nm [29]. This agent reduced the infarct size in a mouse model of myocardial ischemia/reperfusion injury to the same extent as that of unfractionated conditioned medium [29]. Using electron microscopy, ultracentrifugation studies, mass spectrometry, and biochemical assays we subsequently established that this complex was an exosome as it carried exosome-associated markers [29] and had an endosomal origin [57]. Furthermore, the RNA cargo was relatively resistant to RNAse degradation unless the EVs were pretreated with phospholipase or detergent to solubilize the lipid membrane [58]. Thus, the renal protective EVs reported by Bruno *et al.* [56] were different from the cardioprotective EVs [29].

In the last 2 years MSC EVs have been reported to be efficacious in an increasing number of animal models of human diseases, including acute kidney injury [56], cutaneous wound healing [59], cerebral ischemia [60], liver fibrosis and injury [61,62], and hypoxic pulmonary hypertension [63]. Recently, MSC exosomes were administered to a person with treatment-refractory grade IV (severe) acute graft-versus-host disease who went into remission with a dramatic alleviation of her disease symptoms and who remained stable for 5 months [64].

## 20.2 Extracellular vesicles

EVs are membrane vesicles that are released by many cell types into the extracellular space and consist of several classes, including exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, or apoptotic bodies [65]. However, the defining parameters for each of these different classes are not definitive, and the use of the terms exosomes, microvesicles, and microparticles are often ambiguous and not rigorously qualified. Generally, EVs can be formed by either inward budding of endolysosomal vesicles followed by exocytosis (e.g., exosomes), or shedding from the plasma membrane (e.g., microvesicles). They have attracted much interest from the biomedical research community because of their potential as sources of biomarkers for diseases, therapeutic agents, or vehicles for drug delivery. Generally, EVs carry a cargo of proteins and nucleic acids that

reflects their cell of origin and the pathophysiological state of the cell sources. Thus, these proteins or nucleic acids could be used as biomarkers to diagnose, prognosticate, or predict disease and its progression. In addition, these EVs with their cargoes could be internalized by other cells as a form of intercellular communication, making these vesicles potential therapeutic agents or delivery vehicles of therapeutic agents [6,7,66,67].

### 20.2.1 Exosome biology and general functions

The exosome is the best characterized of all the EVs described to date and can be differentiated from other EVs by its biogenesis. The exosome is the only EV known to be derived from endosome, and this endosomal origin of exosomes is reflected in its membrane, which is enriched for lipids commonly found in lipid rafts such as cholesterol, sphingomyelin, and ceramide. Lipid rafts are specialized microdomains in the plasma membrane where much of the cellular endocytosis takes place to form endosomes. Subsequent invaginations of the endosomal membrane generate the numerous intraluminal vesicles (ILVs) within the multivesicular body (MVB). If the MVB fuses with the plasma membrane, the ILVs are released as exosomes [68]. The process by which cellular proteins and RNAs are selected and loaded into exosomes has not been elucidated. While ESCRT (endosomal sorting complex required for transport) has been implicated [69], some proteins are loaded into exosomes in ESCRT-independent higher order oligomerization or a ceramide-dependent process [70,71]. This highly regulated and energy-intensive biogenesis of exosomes indicates their biological importance.

Although the distinguishing feature of the exosome is its endosomal biogenesis, this feature is seldom used as a defining parameter for exosomes, since this is technically challenging. In practice, exosomes are usually defined by technically more amenable parameters, such as a diameter of 40–100 nm, a flotation density of 1.10–1.18 g/mL in a sucrose gradient, and exosome-associated markers such as programmed cell death 6-interacting protein (PDCD6IP, also known as Alix), TSG101, and the tetraspanins, CD9, CD63, or CD81 [72,73]. Recently, we demonstrated using pulse-chase experiments that endocytosed cholera toxin-B chains (CTBs) are released with EVs carrying exosome-associated markers. This is significant as CTB is a pentamer with a high binding affinity for GM1 gangliosides, and GM1 gangliosides are enriched in lipid rafts, which are microdomains of active endocytosis. Thus, CTB-binding activity is a robust marker for the endosomal origin of MSC exosomes, and possibly exosomes from other cell sources as well [57]. As assaying for CTB affinity is technically simple, the endosomal origin of exosomes can be easily validated.

Besides MSCs [29], many other cell types are also known to secrete exosomes. The earliest cell types reported to secrete exosomes were generally, but not exclusively, hematopoietic cells such as B cells [74], dendritic cells [75], mast cells [76], T cells [77], and platelets [78]. Additional cell types included Schwann cells [79], tumor cells [80], skeletal muscle cells [81], human embryonic kidney cells [82], various cancer cell lines [83], and sperm [84]. As a reflection of the diversity in exosome-secreting cell types, exosomes are reportedly found in nearly all extracellular fluids in the body, including bronchial lavage fluid [85], urine [86,87], saliva [88], breast milk [89], and blood [87]. However, it should be noted that the term "exosome" as used in many of these reports could be a mixture of different EV classes that may or may not include bona fide exosomes. Hence, it is possible that exosomes are in fact secreted by a limited number of cell types. To test this possibility we previously evaluated the production of $CD81^+$ CTB-binding vesicles in culture medium conditioned by several cell lines, including the murine ESC-derived insulin-producing cell line ERoSHK6 [90], the human acute monocytic leukemia cell line THP-1, the human embryonic kidney cell line HEK, primary human small airway epithelial cells, and primary human myoblasts, and myotubes differentiated from them. All the lines tested produced exosomes, but in different quantities [66]. The diversity in exosome-secreting cell types indicates that exosome secretion is a conserved cellular function; therefore, exosomes must have important biological functions. When first discovered, exosomes were thought to facilitate disposal of redundant proteins [91], but they are now generally recognized as conserved vehicles for intercellular communication in many biological systems. For example, dendritic cells when pulsed with tumor peptides secrete exosomes to mediate tumor suppression [92], neurons secrete exosome for neurotransmission [93,94], oligodendrocytes secrete exosomes to inhibit myelin membrane biogenesis [95], and exosomes from cardiomyocyte progenitor cells induce migration of endothelial cells and provide cardioprotection against ischemia/reperfusion injury [96–98].

As a conserved mode of intercellular communication to transfer materials from one cell to another, exosomes are inevitably prone to being hijacked by pathogens or diseased cells as vehicles to disseminate disease. Examples to date of exosomes being implicated as carriers of pathogenic materials from diseased or infected cells to disseminate disease include cancer (reviewed in [99]), neurodegenerative diseases such as Parkinson's and Alzheimer's diseases (reviewed in [100]), transmissible spongiform encephalopathies (reviewed in [100]), viral infections (reviewed in [101]), and bacterial infections (reviewed in [102]).

## 20.3 The therapeutic use of exosomes

The conventional forms of cell signaling are autocrine (signaling to a receptor on the same cell that secreted the signal), juxtacrine (cell contact-dependent signaling), paracrine (signaling to a nearby cell), endocrine (signaling by a hormone secreted by an endocrine gland into the bloodstream which carries the hormone to its target cells/tissues elsewhere in the body) and exocrine (secretion of a substance through a duct such as the salivary glands and sweat glands). Exosome-mediated intercellular communication differs from these types of intercellular

communications in that an exosome carries a multimolecular cargo of proteins, RNAs. and lipids that can simultaneously target multiple molecular targets in diverse processes and cell types. For example, the presence of both cytosolic and membrane proteins confers on exosomes the potential to modulate both extracellular and intracellular activities. This modulation is facilitated by the bilipid membrane of exosomes, which not only stabilizes the structural configuration, and thus bioactivity of membrane proteins, but also facilitates the cellular uptake of luminal proteins or RNA through membrane fusion or endocytosis. Exosomes also have many of the desirable attributes of a liposomal drug delivery vehicle [66,67]. Unlike liposomes, the cell-derived lipid membrane is more biocompatible with the body and thus better tolerated or internalized by the body's cells. For example, exosomes derived from dendritic cells can modulate immune responses by transferring peptide-loaded major histocompatibility complex (MHC) class I and II complexes to dendritic cells [103,104]. Exosomes have also been shown to mediate intercellular transfer of mRNAs and microRNAs that resulted in the translation of the transferred mRNA in the recipient cells [105]. We have demonstrated that MSC exosomes are internalized by H9C2 cardiomyocytes [58]. Exosomes also possess the ability to home to specific cell types. For example, melanoma exosomes have been reported to home preferentially to sentinel lymph nodes to promote tumor metastasis [106]. Their membranes could also be modified to enhance cell-type-specific targeting. For example, overexpression of a fusion gene consisting of a neuron-specific rotavirus G (RVG) encoding sequence and a lysosomal-associated membrane protein (LAMP2B) sequence encoding an exosomal membrane protein in dendritic cells resulted in the secretion of exosomes with RVG peptide on their membranes. These exosomes, after loading with small interfering RNA (siRNA), could cross the blood–brain barrier to knock down more than 60% of the siRNA-targeted gene in neurons, microglia, oligodendrocytes, and their precursors [107]. Together, these attributes of exosomes have positioned them as a promising "first-in-class" therapeutic that could potentially manipulate the activities of any cell type in the body through the introduction of exogenous cargo.

### 20.3.1 Mesenchymal stromal cell exosomes

Of all EVs known to be secreted by specific cell types, exosomes secreted by MSCs are currently the best characterized in terms of their therapeutic potential. A large part of this can be attributed to the fact that MSCs are already being used to treat an increasingly wide spectrum of diseases in animal models. More importantly, they are also one of the most clinically evaluated cell types, as evidenced by the increasing number of FDA IND submissions for them, as discussed earlier. This increased scrutiny has led to the redundancy of the differentiation-based rationale for therapeutic efficacy of MSC therapy and the emergence of a secretion-based rationale, again as discussed earlier. This rationale has evolved to an even more specific secreted entity: EVs (reviewed in [7]).

The distillation of the therapeutic potential of MSC EVs represents a logical strategy to efficiently functionalize the role of MSCs as a generic stromal support cell for the maintenance of tissue homeostasis. Such a role of MSCs is consistent with their ubiquitous presence in almost all tissues [108]. Their role as a stromal support cell is best exemplified by their maintenance of homeostasis in the hematopoietic stem cell niche in the bone marrow, such that the quiescence of hematopoietic stem cells, their self-renewal, and differentiation are in equilibrium with the needs of the body [109] (see Chapter 32), as well as in the tumor microenvironment to maintain tumor growth and dissemination (reviewed in [110]).

Tissue homeostasis is an exact state that is very susceptible to the highly dynamic and rapidly evolving fluctuations of the local tissue microenvironment. Its maintenance thus requires timely and appropriate tissue responses. In this regard, MSCs – through the secretion of exosomes with a huge cargo of diverse and biologically active molecules that includes both common exosome proteins and MSC-specific proteins [7] – are well equipped to mediate a stromal support function that induces a timely and appropriate response in the target cells as well as to directly modulate the microenvironment through proteins localized on the exosome membrane or through the release of luminal proteins. Tissue homeostasis is critical to proper and efficient tissue function and is often disrupted in disease or injury. The survival of the tissue, recovery of normal tissue function, and initiation of repair are contingent on the timely restoration of tissue homeostasis. Prolonged delay can lead to irreversible tissue damage. In this regard, EVs/exosomes carrying a complex cargo of biologically active materials encapsulated within a receptor-studded lipid membrane represent the most efficient means to effect a timely restoration of tissue homeostasis. Hence, we postulate that MSCs exert their stromal support function through their exosomes to restore tissue homeostasis to diseased and injured tissues and to facilitate tissue repair and regeneration. This generic mechanism of action could rationalize the efficacy of MSCs in a wide spectrum of diseases and tissue injuries. It could also rationalize the contradicting observations that MSC exosomes both support [111–113] and suppress [114–116] tumor growth, tumor survival, and metastasis. Depending on the stage of tumor growth and the host's response to it, restoration of homeostasis to a tumor microenvironment by MSC exosomes may alleviate host-mediated disruption of the tumor microenvironment and support tumor growth, or abrogate tumor-mediated disruption to restore normal tissue homeostasis and enhance the host's antitumor response.

Evaluation of this hypothesis requires elucidation of the mechanism of action in MSC exosome-mediated therapy. The first premise for such a hypothesis must be the presence of a biochemical potential that could restore homeostasis in fundamental biological processes, and this would in turn require the characterization of the MSC exosome, its cargo, and its biochemical potency.

### 20.3.2 Characterization of mesenchymal stromal cell exosomes

Biochemically, the MSC exosome is a bilipid membrane vesicle with a receptor-studded membrane and luminal cargo of proteins and RNA. While the composition of the MSC exosome determines its biological potency, the MSC exosome should be viewed as a single biological entity and its function is more than a sum of its parts. When this entity is compromised, as in physically disrupted MSC exosomes, they are no longer cardioprotective [117].

As a first-principle approach to elucidate the biological activity of MSC exosomes, we interrogated the biochemistry of the MSC exosome. We had previously demonstrated that the MSC exosome has a cholesterol-rich phospholipid membrane [118] and that this membrane is derived from lipid rafts, which are relatively rigid cholesterol-rich microdomains in the plasma membrane [57]. Lipid rafts are microdomains in the plasma membrane, where most of the cellular endocytic activity is localized, and are enriched in rare phospholipids (such as GM1 gangliosides) and receptors (such as transferrin receptors). By pulse–chase experiments, we have demonstrated that MSC exosomes are derived from GM1-ganglioside-rich and transferrin-receptor-rich lipid rafts in the plasma membranes. Labeled ligands such as CTB (which binds GM1 gangliosides) or transferrin (which binds transferrin receptors) can be chased into exosomes after a short pulse [57].

We have also characterized the protein and RNA cargo of MSC exosomes. The proteins and RNAs are protected by a phospholipid membrane and are relatively resistant to enzymatic degradation by protease or RNAse [58,118]. This resistance is abrogated by pretreatment with phospholipase or detergent. Proteomic analysis of MSC exosomes by mass spectrometry and antibody arrays revealed a large diverse protein cargo of more than 800 unique proteins (www.exocarta.org) [7,119]. To ensure a better coverage of the exosome proteome, mass spectrometry, which has a limited dynamic range of detection, had to be complemented with antibody arrays to detect known proteins or peptides that are too small to be efficiently detected by mass spectrometry.

The RNA cargo of MSC EVs has been analyzed using microarray hybridization [58], polymerase chain reaction [120], and sequencing [121]. The coverage by the former two methods is limited to known RNAs, and both studies focused primarily on microRNAs. The first study reported the enrichment of pre-microRNAs in MSC exosomes and the absence of RNA-induced silencing complex (RISC)-associated proteins [58]. The second study using MSC microvesicles showed that, in addition to microRNAs, MSC microvesicles also contain proteins that are involved in the stability, transport, and processing of both mRNAs and microRNAs. Of the three methods, sequencing, which provides the best coverage, revealed the presence of mRNA and microRNAs in MSC exosomes.

### 20.3.3 The biochemical potential of mesenchymal stromal cell exosomes

The MSC exosome was found to contain at least 800 proteins (www.exocarta.org) [119] and >150 microRNAs [58]. The elucidated proteome of MSC exosomes was diverse and had a vast functional distribution. Collectively, the exosomal proteins were computationally predicted to be active in a wide array of biochemical and cellular processes, including communication, structure and mechanics, inflammation, exosome biogenesis, tissue repair and regeneration, and metabolism. Many of the proteins were biochemically active enzymes or enzyme complexes. The diversity of protein functions could potentially underpin the widely ranging therapeutic efficacy of MSCs, while their distribution across broad ranging cellular functions could provide the biochemical bandwidth to act against diverse and complex diseases or tissue injuries. At this time, the RNA composition of MSC exosomes has not been sufficiently characterized for meaningful analysis. Nevertheless, we note here that, unlike other exosomes reported to date, MSC exosomes are significantly enriched for precursor microRNAs and do not contain any of the RISC components, such as Dicer and Ago2 [58]. (Dicer, also known as endoribonuclease Dicer or helicase with RNase motif, is an enzyme that is part of the RNase III family. Dicer cleaves double-stranded RNA and pre-microRNA into short double-stranded RNA fragments called siRNAs and microRNAs respectively. Ago2 is a member of the Argonaute protein family, which are key players in gene-silencing pathways guided by small RNAs. Small RNAs, such as siRNAs, microRNAs, or Piwi-interacting RNAs, are anchored into specific binding pockets and guide Argonaute proteins to target mRNA molecules for silencing or destruction.)

This preferential sequestering of pre-microRNAs in exosomes demonstrated that the loading of microRNAs into MSC exosomes involved a high degree of regulation. In the absence of RISC elements, the enrichment of pre-microRNA ensures that, upon uptake by recipient cells, exosomes have the potential to exert microRNA-mediated biological activity through the uploading of pre-microRNAs on to the RISC.

Exosome-associated proteins (defined as those proteins found in at least 50% of exosomes) that have been characterized to date include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), pyruvate kinase (PK), eukaryotic translation elongation factor 1A1, milk fat globule epidermal growth factor factor 8 protein, tetraspanins, 14-3-3 proteins, Gα proteins, clathrin, PDCD6IP (also known as Alix), MHC class I, annexins, Rab proteins, the ERM family of proteins (ezrin, radixin, and moesin), actin, tubulin, and heat shock proteins 70 and 90 [7,122].

When analyzed by PANTHER (protein analysis through evolutionary relationships) [123,124], the proteins in the MSC exosome could be functionally clustered into 32 overrepresented and 3 underrepresented biological processes [7,119]. A significant number of the processes, such as the trans-Golgi network, intracellular protein trafficking, general vesicle transport, endocytosis, receptor-mediated endocytosis, other protein targeting

and localization, and exocytosis, are associated with the biogenesis and release of exosomes.

Exosomes are formed through the invagination of the endosomal membranes to form ILVs in the MVBs. The fusion of MVBs with the plasma membrane results in the release of ILVs as exosomes. The mechanism of sorting of proteins, membranes, and possibly RNAs into endosomes and then laterally into ILVs has not yet been well defined. Both ESCRT and ESCRT-independent processes, such as higher order oligomerization or a ceramide-dependent process [70,125], have been implicated in the sorting of exosomal proteins (reviewed in [68]). The presence of ESCRT-associated proteins in MSC exosomes [7,119] and the dependence of MSC exosome production on sphingomyelinase [57] suggest that the loading of the MSC exosome is ESCRT- and ceramide-dependent.

There were also many processes revealed in the analysis of the MSC exosome that are relevant to intercellular communication, cellular motility, inflammation, development and differentiation of mesodermal and ectodermal tissues, including skeletal development and angiogenesis, and fundamental catabolic and anabolic metabolic processes, such as glycolysis. These processes are highly relevant to the maintenance of the tissue microenvironment and cellular homeostasis since they are usually activated or compromised during tissue injury from disease or trauma. These processes need to be restored to baseline homeostasis for the return to normalcy. An interesting feature of the MSC exosome is the underrepresentation of biological processes involved in regulating gene expression at the transcriptional level, suggesting that the MSC exosome probably does not exert its effect through gene expression.

### 20.3.4 Biochemical potency

The computationally predicted processes based on the proteome of exosomes provide an insight into the biochemical potential of MSC exosomes and generate useful testable hypotheses. Given our initial perception of the exosome as a means for disposal of redundant proteins, there is a possibility that the biochemical potential of exosomes has already been expended prior to their loading into the exosomes. Therefore, the computationally predicted processes had to be biochemically validated for potency in modulating cellular processes. Two of these processes will be discussed later (Figure 20.1).

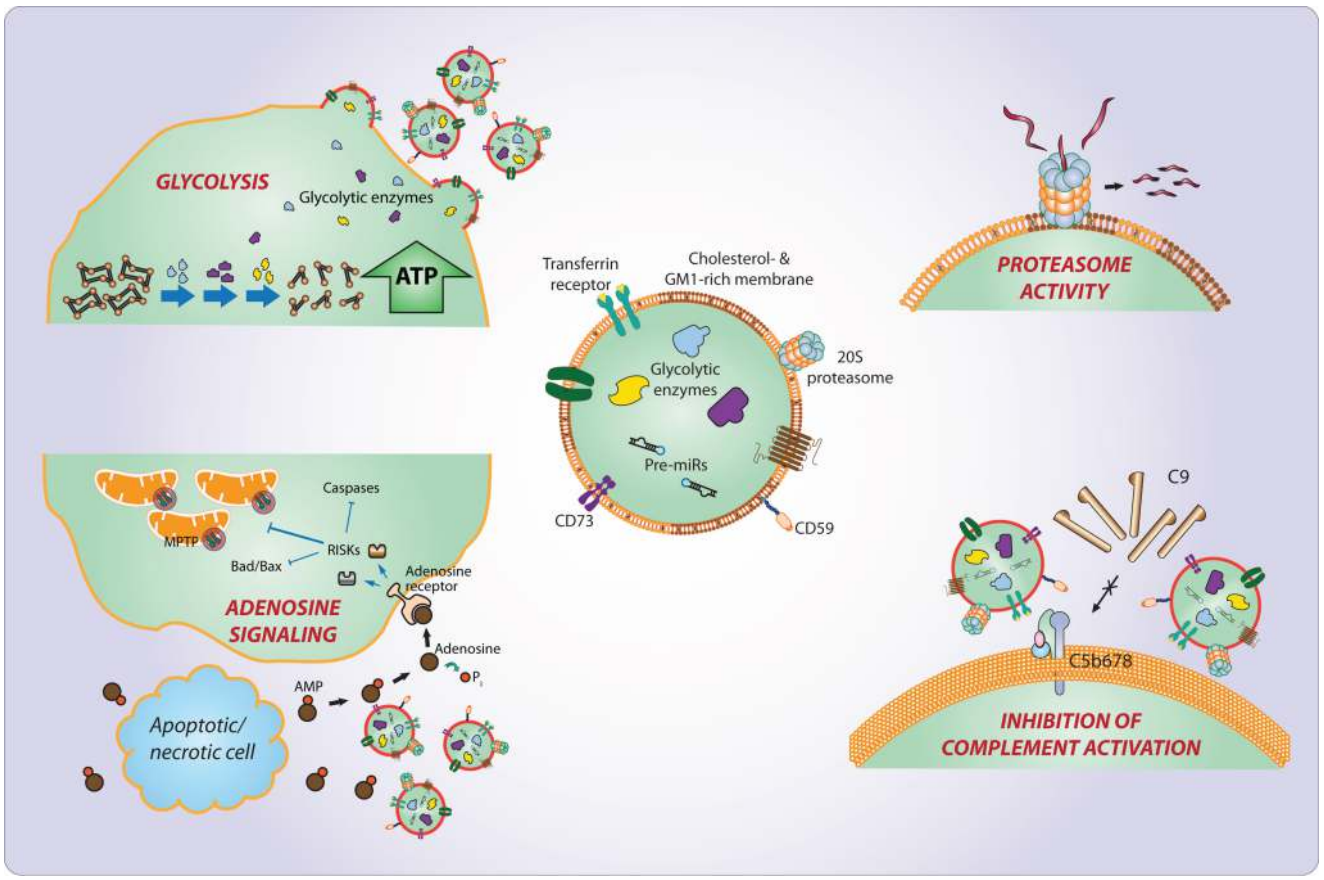



**Figure 20.1** Biochemical potential of MSC exosomes. MSC exosomes contain characteristic features, including a cholesterol- and GM1-rich membrane, transferrin receptors and microRNAs (miRNAs) predominantly in the precursor forms. In addition, they also contain glycolytic enzymes, 20S proteasome, CD73, and CD59, which play key roles in mediating bioenergetics homeostasis, immunoproteasome activity, adenosine-mediated survival signaling, and complement inhibition respectively. RISKs: reperfusion injury salvage kinases. (See color plate section.)

### 20.3.5 Glycolysis

One computational prediction is the capacity of MSC exosomes to drive glycolysis, a fundamental cellular process that is critical in the production of adenosine-5′-triphosphate (ATP) and intermediate metabolites for growth, proliferation, and repair. In MSC exosomes all five enzymes that drive ATP production in glycolysis, namely GAPDH, phosphoglycerate kinase (PGK), phosphoglycerate mutase, enolase, and PK isozyme M2 (PKm2) were present. Importantly, the three enzymes that generate either ATP or the reduced form of nicotinamide adenine dinucleotide (NADH) (GAPDH, PGK, and PKm2) were also enzymatically active [7]. When MSC exosomes were incubated with H9C2 cells that had been treated with oligomycin to inhibit mitochondrial ATPase and oxidative phosphorylation [126], an increased intracellular ATP level was observed within minutes, possibly through glycolysis. This capacity to increase glycolysis has an additional implication as glycolytic intermediates are essential in anabolic reactions to generate biomass for growth and repair (reviewed in [127]) and are critical in anabolic activities to rebuild damaged tissues.

### 20.3.6 Proteasome activity

Proteasomes are protein complexes inside all eukaryotes and archaea (single-cell microorganisms) and in some bacteria. In eukaryotes they are located in the nucleus and the cytoplasm. The main function of the proteasome is to degrade unneeded or damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. Enzymes that help such reactions are called proteases. Proteasomes are part of a major mechanism by which cells regulate the concentration of particular proteins and degrade misfolded proteins. The proteasome subcomponents are often referred to by their Svedberg sedimentation coefficient (denoted *S*). The proteasome most exclusively used in mammals is the cytosolic 26S proteasome, which is about 200 kDa in molecular mass containing one 20S protein subunit and two 19S regulatory cap subunits. Immunoproteasomes contain replacements for the three catalytic subunits of standard proteasomes. In most cells, oxidative stress and proinflammatory cytokines are stimuli that lead to elevated production of immunoproteasomes. Immune system cells, especially antigen-presenting cells, express a higher basal level of immunoproteasomes.

The MSC exosome carries all seven α and seven β chains of the 20S proteasome, and the three β subunits of "immunoproteasome," but not the components of the 26S proteasome [119], suggesting that it carries functional 20S proteasome and immunoproteasome, which is the catalytic core of the 26S proteasome. Consistent with this, MSC exosomes exhibited proteolytic activity that was sensitive to lactacystin, a proteasome-specific inhibitor [119].

### 20.3.7 Signaling: adenosine signaling

One of the characteristic surface markers of MSCs is CD73, which is also found on its exosomes as well as exosomes secreted by other cell types. CD73 is an ecto-5′-nucleotidase and is the only enzyme known to hydrolyze extracellular adenosine monophosphate (AMP) to adenosine [128]. Adenosine is a key activator of extracellular signal-regulated kinase (ERK) and AKT (protein kinase B) phosphorylation and plays a nonredundant role in attenuating inflammation and tissue damage (reviewed in [129]). In mammals, adenosine is produced by the degradation of extracellular ATP and adenosine diphosphate (ADP) that are released during tissue trauma (reviewed in [130]) and has a half-life of <10 s [131]. CD73 in the MSC exosome is enzymatically active, and, in the presence of AMP, MSC exosomes induced phosphorylation of AKT and ERK through a theophylline-sensitive receptor [7].

### 20.3.8 Inhibition of complement activation

CD59 is a small glycosylphosphatidylinositol (GPI)-anchored glycoprotein that plays a crucial role in preventing autologous complement-mediated lysis [132]. It is not only widely expressed in most tissues and in all circulating cells, but is also expressed on exosomes produced by many cell types [133]. The complement system, which consists of at least 30 serum proteins, is a first line of innate immune defense that clears invading microorganisms and cellular debris and activates proinflammatory responses (reviewed in [134], and see Chapter 33). Upon activation the complement system initiates a proteolytic cascade that generates several key defense products, such as C3b, C5a and C5b. This cascade culminates in the formation of the membrane attack complex (MAC) consisting of C5b, C6, C7, C8, and polymeric C9. MAC deposition on target cells results in the formation of a transmembrane channel to cause cell lysis. This lysis is inhibited in cis by CD59 present on the cell membrane [135]. The MSC exosome can inhibit MAC-mediated cell lysis, and this inhibition is abrogated by neutralizing CD59 antibody, suggesting that the MSC exosome can exert a CD59-mediated inhibition of MAC in a trans-acting manner. This possibility is supported by observations that soluble recombinant CD59 could also inhibit MAC formation, albeit less efficiently than GPI-linked CD59 [136,137].

### 20.3.9 Restoring homeostasis

Although MSC exosomes have the biochemical potential to modulate many processes, their efficacy in effecting the stromal support functions of MSCs and maintaining a dynamic homeostatic tissue microenvironment also depends on their versatility and capacity to interact with multiple cell types, both within their immediate vicinity and in remote areas, to elicit appropriate cellular responses.

It is unknown if the MSC exosome cargo resides in one exosome type or is distributed across several exosome or EV types. Additionally, a key requisite of the MSC exosome in mediating the role of MSCs as stromal support cells in order to maintain tissue homeostasis and to respond to the external environment is the capacity to calibrate their biological activity. Based on the enzyme-rich proteome of MCS exosomes, we have previously proposed that much of the therapeutic activity of the MSC exosome is enzymatic [7]. This has critical implications in

maintaining homeostasis, as enzymatic activity can be calibrated (i.e., amplified or inhibited) according to the microenvironment. When a tissue is in homeostasis most biological processes, such as substrates and products, would be in equilibrium, such that additional enzymes would not affect the rate of processes. During injury, when there are changes in substrate/product concentration, pH, or temperature, such that equilibrium is lost and homeostasis is disrupted, the rate of process through modulation of enzyme activity will be altered until equilibrium is restored. The degree of modulation would be calibrated to the degree of change, which in turn would be proportional to the severity of the injury. Thus, enzyme-based activity could potentially elicit a scalable biological response to restore homeostatic equilibrium and as such, mitigate the risk of overdosing or underdosing.

In addition to their biochemical potency, MSC exosomes have the capacity to protect and deliver molecules to the appropriate targets. By encapsulating enzymes within the exosomal membrane, exosomes not only protect their protein and RNA cargo against degradation, but also facilitate the intracellular uptake of enzymes through endocytosis of exosomes by the target cells [58]. We have previously reported that exosome proteins and RNAs are protected against proteinase activity and that this protection is abrogated by pretreatment with phospholipase, a cholesterol chelator or detergent [58,118]. The membrane functions beyond its physical role as a protective barrier since physical disruption of the exosomes without compromising the enzymatic activity of its cargo led to the loss of its cardioprotective function [117].

As with MSCs themselves, MSC exosomes are immune privileged. Administration or exposure of MSC exosomes to animals or immune cells did not elicit an inflammatory response [138]. As exosome uptake has been shown to be dependent on intracellular and microenvironmental acidity [139], and as tissue injury is often characterized by tissue acidosis [140–146], exosomes could be preferentially endocytosed by cells in an injured tissue. Thus, exosomes could home to injured tissues. Indeed, we have observed that uptake by MSC exosomes by H9C2 cardiomyocytes is accelerated in acidic culture medium (unpublished data). Furthermore, the small size of exosomes facilitates their transportation through the blood and other biological fluids to interact with cells in a paracrine and endocrine manner. Thus, exosomes have the biophysical capacity to mediate MSC interactions with multiple cell types both intracellularly and extracellularly in both local and remote areas. Next, we illustrate the biochemical and biophysical capability of MSC exosomes in modulating bioenergetic homeostasis and immune homeostasis.

### 20.3.10 Bioenergetic homeostasis

The first reported therapeutic efficacy of MSC exosomes was their amelioration of myocardial ischemia/reperfusion injury [118]. This injury is characterized by significant temporal quantitative changes in the cardiac proteome after 30 min of ischemia and persists for up to 120 min after reperfusion [147]. The most marked changes were associated with ATP production and apoptosis. In the case of ATP production, the ischemic and reperfused cardiac tissues were severely depleted of the rate-limiting enzymes in fatty acid oxidation, glycolysis, and the tricarboxylic acid (Krebs) cycle. As such, ATP production was curtailed during both ischemia and reperfusion, leading to an ATP deficit that was refractory to the restoration of oxygen during reperfusion. We have previously proposed that part of the therapeutic efficacy of MSC exosomes is mediated by restoring bioenergetic homeostasis. This is achieved by proteomic complementation to compensate for the loss of ATP-generating enzymes with ATP-generating glycolytic enzymes [148]. The RNA component of the MSC exosome is not likely to be the active therapeutic agent in ATP-deficient cardiac tissues since RNA generally exerts its biological activity through ATP-dependent RNA translation or degradation. In contrast, glycolytic enzymes are often driven by the relative ratio of substrate to product such that enzymatic activity is often calibrated directly to substrate concentration and inversely to product concentration. Hence, the presence of all enzymes in the ATP-generating phase of glycolysis would enable MSC exosomes to increase glycolytic flux and ATP production until bioenergetics homeostasis is restored. Thus, the concentration of substrates and products reaches equilibrium and the flux moderates to a steady state.

An important caveat in this hypothesis is that proteins in the MSC exosome can be internalized by cardiomyocytes. In general, our hypothesis that MSC exosomes reduced reperfusion injury in ischemic heart tissues by restoring bioenergetics homeostasis is consistent with our experimental observations. Indeed, MSC exosomes are internalized by H9C2 cardiomyocytes [104]. In a mouse model of myocardial ischemia/reperfusion injury, exosome-treated animals exhibited an increased ATP/ADP ratio that was concomitant with improved cardiac function [117].

### 20.3.11 Immune homeostasis

Immune homeostasis refers to physiological equilibrium between attack and defense mechanisms against pathogens, foreign or diseased tissues, and tolerance for self. This equilibrium is maintained by checks and balances involving many immune cell types and soluble mediators. Dysregulation of immune homeostasis compromises the body's defense and self–nonself recognition, leading to disease or autoimmunity.

We have previously demonstrated *in vitro* that MSC exosomes induce polarization of regulatory T cells (Tregs) through the activation of monocytes [138]. BALB/cJ mice ($H2^d$ genotype) were grafted with tail skin from C57BL/6J mice ($H2^b$ genotype, and thus mismatched for all the antigens of the MHC with BALB/cJ mice). The BALB/cJ recipients were then given subcutaneous injections of 0.3 μg exosomes in 50 μl phosphate-buffered saline (PBS) or of 50 μl PBS as control. The exosome-treated and PBS-treated mice took 13 days and 11 days respectively to reject the skin grafts. The level of Tregs was significantly higher in the spleens of exosome-treated recipient animals than in the PBS-treated animals. However, Treg induction was not observed in the spleens of non-grafted mice that were given the same

exosome regimen. Intrasplenic injection of either exosomes or PBS did not induce a higher Treg level in exosome-treated animals. The MSC exosomes induce Tregs only when the immune system is activated. Since Tregs are immunosuppressive, the induction of Tregs by MSC exosomes in an activated immune system demonstrated that MSC exosomes can attenuate immune activity and enhance the restoration of immune homeostasis. Conversely, as MSC exosomes did not elicit Treg induction in mice that had not been immunologically challenged, MSC exosomes do not induce immune suppression and disrupt immune homeostasis in normal animals.

In summary, MSC exosomes induced expansion of Tregs in animals whose immune system was stimulated. In animals without immune stimulation, MSC exosomes had no effect on Treg expansion. Thus, MSC exosomes are not immunosuppressive but are immune modulatory.

## 20.4 The clinical translation of mesenchymal stromal cell exosomes

The discovery that MSC therapeutic activity is mediated by exosomes could potentially transform MSC therapy from a cell-based approach to a biologic-agent-based approach. This change has wide-ranging ramifications. Cell-based therapies pose significant risks and challenges in manufacture, storage, delivery, safety, and regulation. Eliminating these requirements would reduce the complexity of the manufacturing, processing, and storage processes, resulting in increased reproducibility, lower costs, and better quality control and assurance. The risks inherent in the use of stromal cells include the risk of occlusion in the microvasculature after intravenous or intra-arterial administration, which can cause embolism and death [149]. Theoretically, differentiation of MSCs into inappropriate cell types could cause complications such as tumor development, immune rejection, proarrhythmic side effects [150–152], or ectopic calcifications [153]. In practice, however, these complications have not occurred in human recipients of MSCs administered either intravenously or intra-arterially.

MSC exosomes being part of MSC biology cannot pose greater risks than MSCs themselves. Their small size mitigates some of the theoretical risks of MSC administration. It is possible that their use would be safer and cheaper than MSCs.

An important factor in the translation of MSC exosome into the clinic is the manufacturing process and the most important requisite is the cell source. Ideally, the cell source would be a high exosome-yielding clonal cell line with a very large expansion potential to ensure reproducible cost-effective production of exosomes. Exosome production by MSCs is inversely correlated with the age of the MSC donor, such that MSCs derived from human embryonic stem cells produce the most exosomes per cell, followed by those derived from fetal tissues, umbilical cord, and adult bone marrow [154]. Human MSCs derived from embryonic stem cells or induced pluripotent stem cells (see Chapter 31) may the best candidate cell source for exosome production. Additionally, human embryonic stem-cell-derived MSCs can be immortalized with a *MYC* transgene and cloned without compromising the yield or quality of the exosomes [155], although the use of a potential oncogene for this purpose would be unacceptable to both clinicians and regulatory authorities. Notably, however, MSC exosomes do not have detectable MYC protein or RNA, which is consistent with the general lack of transcription factors in the proteome of exosomes [24].

## 20.5 Conclusions

Through the iterative process of scientific enquiry in animal models and clinical studies, the use of MSCs for potential treatment purposes has been evolving to a cell secretion-based approach. Among the many candidates proposed as the therapeutic agent in MSC secretions, the MSC exosome has emerged as a promising candidate. Based on the biochemical potency of its diverse cargo of proteins and RNA, MSC exosomes could underpin the potential efficacy of MSCs against diverse and complex diseases by functionalizing the stromal support role of MSCs in restoring and maintaining tissue homeostasis. The use of MSC exosomes as therapeutic agents instead of cells may simplify MSC-based therapy across the entire value chain of manufacturing, storage, delivery, and regulatory oversight. This could lead to a more affordable drug. The use of exosomes also mitigates the risks associated with the transplantation of cells. Nevertheless, the clinical development of the MSC exosome as a drug faces challenges in manufacturing and regulatory oversight that are unique to a potential first-in-class drug.

## References

1 M. Mendicino, A. M. Bailey, K. Wonnacott, *et al.* MSC-based product characterization for clinical trials: an FDA perspective. *Cell Stem Cell* 2014;**14**(2):141–145.
2 A. Giordano, U. Galderisi, I. R. Marino. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. *J Cell Physiol* 2007;**211**(1):27–35.
3 A. J. Friedenstein, K. V. Petrakova, A. I. Kurolesova, G. P. Frolova., Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 1968;**6**(2):230–247.
4 G. Brooke, M. Cook, C. Blair, *et al.* Therapeutic applications of mesenchymal stromal cells. *Semin Cell Devl Biol* 2007;**18**(6): 846–858.
5 M. Pittenger, A. Mackay, S. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.
6 R. C. Lai, T. S. Chen, S. K. Lim. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* 2011;**6**(4):481–492.
7 R. Lai, R. Yeo, S. Tan, *et al.* Mesenchymal stem cell exosomes: the future MSC-based therapy? In: Chase LGV, Mohan C (eds), *Mesenchymal Stem Cell Therapy*. Humana Press, 2013; pp. 39–62.

8 J. Ferrand, D. Noel, P. Lehours, *et al.* Human bone marrow-derived stem cells acquire epithelial characteristics through fusion with gastrointestinal epithelial cells. *PLoS ONE* 2011;**6**(5):e19569.

9 J. L. Spees, S. D. Olson, J. Ylostalo, *et al.* Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma. *Proc Natl Acad Sci U S A* 2003;**100**(5):2397–2402.

10 G. Vassilopoulos, P. R. Wang, D. W. Russell. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 2003;**422**(6934): 901–904.

11 D. G. Phinney, D. J. Prockop. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* 2007;**25**(11): 2896–2902.

12 Y. Iso, J. L. Spees, C. Serrano, *et al.* Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochem Biophys Res Commun* 2007;**354**(3):700–706.

13 L. da Silva Meirelles, A. I. Caplan, N. B. Nardi. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 2008;**26**(9): 2287–2299.

14 W. Dai, S. L. Hale, B. J. Martin, *et al.* Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation* 2005;**112**(2):214–223.

15 N. Noiseux, M. Gnecchi, M. Lopez-Ilasaca, *et al.* Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther* 2006;**14**(6):840–850.

16 A. I. Caplan, J. E. Dennis. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006;**98**(5):1076–1084.

17 S. E. Haynesworth, M. A. Baber, A. I. Caplan. Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1α. *J Cell Physiol* 1996;**166**(3): 585–592.

18 T. Kinnaird, E. Stabile, M. S. Burnett, S. E. Epstein. Bone-marrow-derived cells for enhancing collateral development: mechanisms, animal data, and initial clinical experiences. *Circ Res* 2004;**95**(4): 354–363.

19 D. Patschan, M. Plotkin, M. S. Goligorsky. Therapeutic use of stem and endothelial progenitor cells in acute renal injury: ça ira. *Curr Opin Pharmacol* 2006;**6**(2):176–183.

20 Y. Miyahara, N. Nagaya, M. Kataoka, *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* 2006;**12**(4):459–465.

21 J. Y. Min, M. F. Sullivan, Y. Yang, *et al.* Significant improvement of heart function by cotransplantation of human mesenchymal stem cells and fetal cardiomyocytes in postinfarcted pigs. *Ann Thorac Surg* 2002;**74**(5):1568–1575.

22 T. Kinnaird, E. Stabile, M. S. Burnett, *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ Res* 2004;**94**(5):678–685.

23 M. Gnecchi, H. He, N. Noiseux, *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* 2006;**20**(6):661–669.

24 S. K. Sze, D. P. de Kleijn, R. C. Lai, *et al.* Elucidating the secretion proteome of human embryonic stem cell-derived mesenchymal stem cells. *Mol Cell Proteomics* 2007;**6**(10):1680–1689.

25 L. Timmers, S.-K. Lim, F. Arslan, *et al.* Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2008;**1**:129–137.

26 M. Gnecchi, H. He, O. D. Liang, *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;**11**(4):367–368.

27 M. Mirotsou, Z. Zhang, A. Deb, *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* 2007;**104**(5):1643–1648.

28 L. Timmers, S. K. Lim, F. Arslan, *et al.* Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;**1**(2):129–137.

29 R. C. Lai, F. Arslan, M. M. Lee, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**(3):214–222.

30 R. C. Lai, F. Arslan, S. S. Tan, *et al.* Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J Mol Cell Cardiol* 2010;**48**(6):1215–1224.

31 B. Bi, R. Schmitt, M. Israilova, *et al.* Stromal cells protect against acute tubular injury via an endocrine effect. *J Am Soc Nephrol* 2007;**18**(9):2486–2496.

32 B. Parekkadan, D. van Poll, K. Suganuma, *et al.* Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS ONE* 2007;**2**(9):e941.

33 L. Timmers, S. K. Lim, I. E. Hoefer, *et al.* Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res* 2011;**6**(3):206–214.

34 R. Schafer, H. Northoff. Cardioprotection and cardiac regeneration by mesenchymal stem cells. *Panminerva Med* 2008;**50**(1):31–39.

35 R. W. Yeo, R. C. Lai, K. H. Tan, S. K. Lim. Exosome: a novel and safer therapeutic refinement of mesenchymal stem cell. *Exosomes Microvesicles* 2013;**1**(7):1–12.

36 S. Ghannam, C. Bouffi, F. Djouad, *et al.* Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. *Stem Cell Res Ther* 2010;**1**(1):2.

37 I. Rasmusson, O. Ringden, B. Sundberg, K. Le Blanc. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005;**305**(1):33–41.

38 H. S. Kang, M. Habib, J. Chan, *et al.* A paradoxical role for IFN-γ in the immune properties of mesenchymal stem cells during viral challenge. *Exp Hematol* 2005;**33**(7):796–803.

39 M. Krampera, L. Cosmi, R. Angeli, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**(2):386–398.

40 R. Meisel, A. Zibert, M. Laryea, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**(12): 4619–4621.

41 K. Sato, K. Ozaki, I. Oh, *et al.* Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007;**109**(1):228–234.

42 F. Gieseke, B. Schutt, S. Viebahn, *et al.* Human multipotent mesenchymal stromal cells inhibit proliferation of PBMCs independently of IFNγR1 signaling and IDO expression. *Blood* 2007;**110**(6):2197–2200.

43 F. Djouad, L. Charbonnier, C. Bouffi, *et al.* Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 2007;**25**:2025–2032.

44 G. Ren, J. Su, L. Zhang, *et al.* Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells* 2009;**27**(8):1954–1962.

45 S. Aggarwal, M. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.

46 D. Chabannes, M. Hill, E. Merieau, *et al.* A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* 2007;**110**:3691–3694.

47 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**: 3838–3843.

48 X. Jiang, Y. Zhang, B. Liu, *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**:4120–4126.

49 A. Nasef, C. Mazurier, S. Bouchet, *et al.* Leukemia inhibitory factor: role in human mesenchymal stem cells mediated immunosuppression. *Cell Immunol* 2008;**253**:16–22.

50 A. Nasef, Y. Zhang, C. Mazurier, *et al.* Selected Stro-1-enriched bone marrow stromal cells display a major suppressive effect on lymphocyte proliferation. *Int J Lab Hematol* 2009;**31**:9–19.

51 K. Nemeth, A. Leelahavanichkul, P. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

52 L. Raffaghello, G. Bianchi, M. Bertolotto, *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 2008;**26**: 151–162.

53 Z. Selmani, A. Naji, I. Zidi, *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**: 212–222.

54 G. Spaggiari, H. Abdelrazik, F. Becchetti, L. Moretta. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin $E_2$. *Blood* 2009;**113**:6576–6583.

55 G. Xu, Y. Zhang, L. Zhang, *et al.* The role of IL-6 in inhibition of lymphocyte apoptosis by mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;**361**:745–750.

56 S. Bruno, C. Grange, M. C. Deregibus, *et al.* Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;**20**(5):1053–1067.

57 S. S. Tan, Y. Yin, T. Lee, *et al.* Therapeutic MSC exosomes are derived from lipid raft microdomains in the plasma membrane. *J Extracell Vesicles* 2013;**2**. http://www.journalofextracellularvesicles.net/index.php/jev/article/view/22614 (accessed February 26, 2016).

58 T. S. Chen, R. C. Lai, M. M. Lee, *et al.* Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res* 2010;**38**(1):215–224.

59 B. Zhang, M. Wang, A. Gong, *et al.* HucMSC-exosome mediated-Wnt4 signaling is required for cutaneous wound healing. *Stem Cells* 2015;**33**(7):2158–2168.

60 H. Xin, Y. Li, Z. Liu, *et al.* MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013;**31**(12):2737–2746.

61 T. Li, Y. Yan, B. Wang, *et al.* Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013;**22**(6):845–854.

62 C. Y. Tan, R. C. Lai, W. Wong, *et al.* Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 2014;**5**(3):76.

63 C. Lee, S. A. Mitsialis, M. Aslam, *et al.* Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 2012;**126**(22): 2601–2611.

64 L. Kordelas, V. Rebmann, A. K. Ludwig, *et al.* MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014;**28**(4):970–973.

65 C. Thery, M. Ostrowski, E. Segura. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009;**9**(8):581–593.

66 R. W. Yeo, R. C. Lai, B. Zhang, *et al.* Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013;**65**(3):336–341.

67 R. C. Lai, R. W. Yeo, K. H. Tan, S. K. Lim. Exosomes for drug delivery – a novel application for the mesenchymal stem cell. *Biotechnol Adv*. 2013;**31**(5):543–551.

68 M. Simons, G. Raposo. Exosomes – vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 2009;**21**(4):575–581.

69 C. Raiborg, T. E. Rusten, H. Stenmark. Protein sorting into multivesicular endosomes. *Curr Opin Cell Biol* 2003;**15**(4):446–455.

70 Y. Fang, N. Wu, X. Gan, *et al.* Higher-order oligomerization targets plasma membrane proteins and HIV gag to exosomes. *PLoS Biol* 2007;**5**(6):e158.

71 K. Trajkovic, C. Hsu, S. Chiantia, *et al.* Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008;**319**(5867):1244–1247.

72 R. Wubbolts, R. S. Leckie, P. T. Veenhuizen, *et al.* Proteomic and biochemical analyses of human B cell-derived exosomes. Potential implications for their function and multivesicular body formation. *J Biol Chem* 2003;**278**(13):10963–10972.

73 A. de Gassart, C. Geminard, B. Fevrier, *et al.* Lipid raft-associated protein sorting in exosomes. *Blood* 2003;**102**(13):4336–4344.

74 G. Raposo, H. W. Nijman, W. Stoorvogel, *et al.* B lymphocytes secrete antigen-presenting vesicles. *J Exp Med* 1996;**183**(3): 1161–1172.

75 L. Zitvogel, A. Regnault, A. Lozier, *et al.* Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nat Med* 1998;**4**(5):594–600.

76 G. Raposo, D. Tenza, S. Mecheri, *et al.* Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Mol Biol Cell* 1997;**8** (12):2631–2645.

77 P. J. Peters, H. J. Geuze, H. A. Van Der Donk, *et al.* Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *Eur J Immunol* 1989;**19**(8): 1469–1475.

78 H. F. Heijnen, A. E. Schiel, R. Fijnheer, *et al.* Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood* 1999;**94**(11):3791–3799.

79 B. Fevrier, D. Vilette, F. Archer, *et al.* Cells release prions in association with exosomes. *Proc Natl Acad Sci U S A* 2004;**101**(26):9683–9688.

80 J. Wolfers, A. Lozier, G. Raposo, *et al.* Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nat Med* 2001;**7**(3):297–303.

81 M. C. Le Bihan, A. Bigot, S. S. Jensen, *et al.* In-depth analysis of the secretome identifies three major independent secretory pathways in differentiating human myoblasts. *J Proteomics* 2012;**77**:344–356.

82 V. Sokolova, A.-K. Ludwig, S. Hornung, *et al.* Characterisation of exosomes derived from human cells by nanoparticle tracking analysis and scanning electron microscopy. *Colloids Surf B Biointerfaces* 2011;**87**(1):146–150.

83 A. Clayton, S. Al-Taei, J. Webber, *et al.* Cancer exosomes express CD39 and CD73, which suppress T cells through adenosine production. *J Immunol* 2011;**187**(2):676–683.

84 R. Sullivan, F. Saez, J. Girouard, G. Frenette. Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood Cells Mol Dis* 2005;**35**(1):1–10.

85 C. Admyre, J. Grunewald, J. Thyberg, *et al.* Exosomes with major histocompatibility complex class II and co-stimulatory molecules are present in human BAL fluid. *Eur Respir J* 2003;**22**(4):578–583.

86 T. Pisitkun, R. F. Shen, M. A. Knepper. Identification and proteomic profiling of exosomes in human urine. *Proc Natl Acad Sci U S A* 2004;**101**(36):13368–13373.

87 M. P. Caby, D. Lankar, C. Vincendeau-Scherrer, *et al.* Exosomal-like vesicles are present in human blood plasma. *Int Immunol* 2005;**17**(7):879–887.

88 E. K. Kapsogeorgou, R. F. Abu-Helu, H. M. Moutsopoulos, M. N. Manoussakis. Salivary gland epithelial cell exosomes: a source of autoantigenic ribonucleoproteins. *Arthritis Rheum* 2005;**52**(5):1517–1521.

89 C. Admyre, S. M. Johansson, K. R. Qazi, *et al.* Exosomes with immune modulatory features are present in human breast milk. *J Immunol* 2007;**179**(3):1969–1978.

90 G. Li, R. Luo, J. Zhang, *et al.* Generating mESC-derived insulin-producing cell lines through an intermediate lineage-restricted progenitor line. *Stem Cell Res* 2009;**2**(1):41–55.

91 B. T. Pan, R. M. Johnstone. Fate of the transferrin receptor during maturation of sheep reticulocytes *in vitro*: selective externalization of the receptor. *Cell* 1983;**33**(3):967–978.

92 D. M. Pegtel, K. Cosmopoulos, D. A. Thorley-Lawson, *et al.* Functional delivery of viral miRNAs via exosomes. *Proc Natl Acad Sci U S A* 2010;**107**(14):6328–6333.

93 J. Faure, G. Lachenal, M. Court, *et al.* Exosomes are released by cultured cortical neurones. *Mol Cell Neurosci* 2006;**31**(4):642–648.

94 G. Lachenal, K. Pernet-Gallay, M. Chivet, *et al.* Release of exosomes from differentiated neurons and its regulation by synaptic glutamatergic activity. *Mol Cell Neurosci* 2011;**46**(2):409–418.

95 M. Bakhti, C. Winter, M. Simons. Inhibition of myelin membrane sheath formation by oligodendrocyte-derived exosome-like vesicles. *J Biol Chem* 2011;**286**(1):787–796.

96 K. R. Vrijsen, J. P. G. Sluijter, M. W. L. Schuchardt, *et al.* Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *J Cell Mol Med* 2010;**14**(5):1064–1070.

97 L. Chen, Y. Wang, Y. Pan, *et al.* Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury. *Biochem Biophys Res Commun* 2013;**431**(3):566–571.

98 A. G. Ibrahim, K. Cheng, E. Marban. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem Cell Rep* 2014;**2**(5):606–619.

99 C. Yang, P. D. Robbins. The roles of tumor-derived exosomes in cancer pathogenesis. *Clin Dev Immunol* 2011;**2011**:842849.

100 L. Rajendran, J. Bali, M. M. Barr, *et al.* Emerging roles of extracellular vesicles in the nervous system. *J Neurosci* 2014;**34**(46):15482–15489.

101 T. Wurdinger, N. N. Gatson, L. Balaj, *et al.* Extracellular vesicles and their convergence with viral pathways. *Adv Virol* 2012;**2012**:767694.

102 J. M. Silverman, N. E. Reiner. Exosomes and other microvesicles in infection biology: organelles with unanticipated phenotypes. *Cell Microbiol* 2011;**13**(1):1–9.

103 C. Thery, L. Duban, E. Segura, *et al.* Indirect activation of naive $CD4^+$ T cells by dendritic cell-derived exosomes. *Nat Immunol* 2002;**3**(12):1156–1162.

104 F. Andre, N. Chaput, N. E. Schartz, *et al.* Exosomes as potent cell-free peptide-based vaccine. I. Dendritic cell-derived exosomes transfer functional MHC class I/peptide complexes to dendritic cells. *J Immunol* 2004;**172**(4):2126–2136.

105 H. Valadi, K. Ekström, A. Bossios, *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;**9**(6):654–659.

106 J. L. Hood, R. S. San, S. A. Wickline. Exosomes released by melanoma cells prepare sentinel lymph nodes for tumor metastasis. *Cancer Res* 2011;**71**(11):3792–3801.

107 L. Alvarez-Erviti, Y. Seow, H. Yin, *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 2011;**29**(4):341–345.

108 M. Dominici. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

109 A. Ehninger, A. Trumpp The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011;**208**(3):421–428.

110 I. A. Droujinine, M. A. Eckert, W. Zhao. To grab the stroma by the horns: from biology to cancer therapy with mesenchymal stem cells. *Oncotarget* 2013;**4**(5):651–664.

111 W. Zhu, L. Huang, Y. Li, *et al.* Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth *in vivo*. *Cancer Lett* 2012;**315**(1):28–37.

112 A. M. Roccaro, A. Sacco, P. Maiso, *et al.* BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest* 2013;**123**(4):1542–1555.

113 J. Wang, A. Hendrix, S. Hernot, *et al.* Bone marrow stromal cell-derived exosomes as communicators in drug resistance in multiple myeloma cells. *Blood* 2014;**124**(4):555–566.

114 M. Katakowski, B. Buller, X. Zheng, *et al.* Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett* 2013;**335**(1):201–204.

115 M. Ono, N. Kosaka, N. Tominaga, *et al.* Exosomes from bone marrow mesenchymal stem cells contain a microRNA that promotes dormancy in metastatic breast cancer cells. *Sci Signal* 2014;**7**(332):ra63.

116 J.-K. Lee, S.-R. Park, B.-K. Jung, *et al.* Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* 2013;**8**(12):e84256.

117 F. Arslan, R. C. Lai, M. B. Smeets, *et al.* Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013;**10**(3):301–312.

118 R. C. Lai, F. Arslan, M. M. Lee, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214–222.

119 R. C. Lai, S. S. Tan, B. J. Teh, *et al.* Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int J Proteomics* 2012;**2012**:971907.

120 F. Collino, M. C. Deregibus, S. Bruno, *et al.* Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PLoS ONE* 2010;**5**(7):e11803.

121 A. Eirin, S. M. Riester, X.-Y. Zhu, *et al.* MicroRNA and mRNA cargo of extracellular vesicles from porcine adipose tissue-derived mesenchymal stem cells. *Gene* 2014;**551**(1):55–64.

122 C. Thery, M. Ostrowski, E. Segura. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009;**9**(8):581–593.

123 P. D. Thomas, M. J. Campbell, A. Kejariwal, *et al.* PANTHER: a library of protein families and subfamilies indexed by function. *Genome Res* 2003;**13**(9):2129–2141.

124 P. D. Thomas, A. Kejariwal, N. Guo, *et al.* Applications for protein sequence–function evolution data: mRNA/protein expression analysis and coding SNP scoring tools. *Nucleic Acids Res* 2006;**34**(Web Server issue):W645–W650.

125 K. Trajkovic, C. Hsu, S. Chiantia, *et al.* Ceramide triggers budding of exosome vesicles into multivesicular endosomes. *Science* 2008;**319**(5867):1244–1247.

126 H. S. Penefsky. Mechanism of inhibition of mitochondrial adenosine triphosphatase by dicyclohexylcarbodiimide and oligomycin: relationship to ATP synthesis. *Proc Natl Acad Sci U S A* 1985;**82**(6):1589–1593.

127 S. Y. Lunt, M. G. Vander Heiden. Aerobic glycolysis: meeting the metabolic requirements of cell proliferation. *Annu Rev Cell Dev Biol* 2011;**27**:441–464.

128 S. P. Colgan, H. K. Eltzschig, T. Eckle, L. F. Thompson. Physiological roles for ecto-5′-nucleotidase (CD73). *Purinergic Signal* 2006;**2**(2):351–360.

129 G. G. Yegutkin. Nucleotide- and nucleoside-converting ectoenzymes: important modulators of purinergic signalling cascade. *Biochim Biophys Acta* 2008;**1783**(5):673–694.

130 J. Luthje. Origin, metabolism and function of extracellular adenine nucleotides in the blood. *Klin Wochenschr* 1989;**67**(6):317–327.

131 G. H. Moser, J. Schrader, A. Deussen. Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* 1989;**256**(4 Pt 1):C799–C806.

132 A. P. Alegretti, T. Mucenic, J. C. T. Brenol, R. M. Xavier. O papel das proteínas reguladoras do complemento CD55/CD59 em células de sangue periférico de pacientes com lúpus eritematoso sistêmico. *Rev Bras Reumatol* 2009;**49**:276–287.

133 H. Kalra, R. J. Simpson, H. Ji, *et al.* Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol* 2012;**10**(12):e1001450.

134 P. F. Zipfel, C. Skerka. Complement regulators and inhibitory proteins. *Nat Rev Immunol* 2009;**9**(10):729–740.

135 A. Davies, D. L. Simmons, G. Hale, *et al.* CD59, an LY-6-like protein expressed in human lymphoid cells, regulates the action of the complement membrane attack complex on homologous cells. *J Exp Med* 1989;**170**(3):637–654.

136 L. M. Li, J. B. Li, Y. Zhu, G. Y. Fan. Soluble complement receptor type 1 inhibits complement system activation and improves motor function in acute spinal cord injury. *Spinal Cord* 2010;**48**(2):105–111.

137 J. Gandhi, S. M. Cashman, R. Kumar-Singh. Soluble CD59 expressed from an adenovirus *in vivo* is a potent inhibitor of complement deposition on murine liver vascular endothelium. *PLoS ONE* 2011;**6**(6):e21621.

138 B. Zhang, Y. Yin, R. C. Lai, *et al.* Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* 2014;**23**(11):1233–1244.

139 I. Parolini, C. Federici, C. Raggi, *et al.* Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;**284**(49):34211–34222.

140 R. van Zwieten, R. Wever, M. N. Hamers, *et al.* Extracellular proton release by stimulated neutrophils. *J Clin Invest* 1981;**68**(1):310–313.

141 N. Borregaard, J. H. Schwartz, A. I. Tauber. Proton secretion by stimulated neutrophils. Significance of hexose monophosphate shunt activity as source of electrons and protons for the respiratory burst. *J Clin Invest* 1984;**74**(2):455–459.

142 O. D. Rotstein, P. E. Nasmith, S. Grinstein. The *Bacteroides* by-product succinic acid inhibits neutrophil respiratory burst by reducing intracellular pH. *Infect Immun* 1987;**55**(4):864–870.

143 O. D. Rotstein, T. Vittorini, J. Kao, *et al.* A soluble *Bacteroides* by-product impairs phagocytic killing of *Escherichia coli* by neutrophils. *Infect Immun* 1989;**57**(3):745–753.

144 M. M. Kopaniak, A. C. Issekutz, H. Z. Movat. Kinetics of acute inflammation induced by *E coli* in rabbits. Quantitation of blood flow, enhanced vascular permeability, hemorrhage, and leukocyte accumulation. *Am J Pathol* 1980;**98**(2):485–498.

145 T. Arnould, R. Thibaut-Vercruyssen, N. Bouaziz, *et al.* $PGF_{2\alpha}$, a prostanoid released by endothelial cells activated by hypoxia, is a chemoattractant candidate for neutrophil recruitment. *Am J Pathol* 2001;**159**(1):345–357.

146 M. J. Grimshaw, F. R. Balkwill. Inhibition of monocyte and macrophage chemotaxis by hypoxia and inflammation – a potential mechanism. *Eur J Immunol* 2001;**31**(2):480–489.

147 X. Li, F. Arslan, Y. Ren, *et al.* Metabolic adaptation to a disruption in oxygen supply during myocardial ischemia and reperfusion is underpinned by temporal and quantitative changes in the cardiac proteome. *J Proteome Res* 2012;**11**(4):2331–2346.

148 R. C. Lai, R. W. Yeo, K. H. Tan, S. K. Lim. Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen Med* 2013;**8**(2):197–209.

149 D. Furlani, M. Ugurlucan, L. Ong, *et al.* Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal

stem cells and intravital microscopy. *Microvasc Res* 2009;**77**(3): 370–376.

150 M. G. Chang, L. Tung, R. B. Sekar, *et al.* Proarrhythmic potential of mesenchymal stem cell transplantation revealed in an *in vitro* coculture model. *Circulation* 2006;**113**(15):1832–1841.

151 H. N. Pak, M. Qayyum, D. T. Kim, *et al.* Mesenchymal stem cell injection induces cardiac nerve sprouting and increased tenascin expression in a swine model of myocardial infarction. *J Cardiovasc Electrophysiol* 2003;**14**(8):841–848.

152 M. J. Price, C. C. Chou, M. Frantzen, *et al.* Intravenous mesenchymal stem cell therapy early after reperfused acute myocardial infarction improves left ventricular function and alters electrophysiologic properties. *Int J Cardiol* 2006; **111**(2):231–239.

153 M. Breitbach, T. Bostani, W. Roell, *et al.* Potential risks of bone marrow cell transplantation into infarcted hearts. *Blood* 2007;**110** (4):1362–1369.

154 T. S. Chen, R. W. Y. Yeo, F. Arslan, *et al.* Efficiency of exosome production correlates inversely with the developmental maturity of MSC donor. *J Stem Cell Res Ther* 2013;**3**(3):145.

155 T. S. Chen, F. Arslan, Y. Yin, *et al.* Enabling a robust scalable manufacturing process for therapeutic exosomes through oncogenic immortalization of human ESC-derived MSCs. *J Transl Med* 2011;**9**(1):47.

CHAPTER 21

# Role of tunneling nanotube crosstalk with distressed cardiomyocytes in controlling the heart repair potential of mesenchymal stromal cells

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## 21.1 Introduction

Cardiovascular diseases are the main cause of death in Western countries. Fundamentally, this is due to the poor ability of dead myocardium to self-regenerate. Cell therapies represent one of the most promising approaches to rebuild the damaged heart, particularly those based on mesenchymal stromal cells (MSCs) owing to their unique properties and clinical safety. Nevertheless, despite a flurry of studies reporting the cardiac functional benefits of MSC delivery in various experimental small and large animal models of myocardial infarction, clinical trials in humans have revealed mixed results, emphasizing the need to maximize the therapeutic effectiveness of MSCs. Gaining new insights into the mechanisms underlying the regenerative effects of MSCs appears to be of critical importance. According to recent studies, these mechanisms seem to be governed by the recipient microenvironment in which MSCs support the cell-to-cell communication between the transplanted MSCs and resident neighboring cardiac cells to play a critical role in the wound healing processes.

In this chapter, after a brief introduction on the potential of MSCs in cardiac cell therapy, we will present an overview on whether cell-to-cell communication of MSCs with distressed cardiomyocytes alters MSC behavior by specially focusing on the role of tunneling nanotube (TNT)-mediated intercellular connections.

## 21.2 Mesenchymal stromal cells as a promising tool to regenerate damaged heart tissue

### 21.2.1 Degenerative cardiac diseases: a major public health problem

Heart disorders are a major health concern worldwide and responsible for millions of death every year. In particular,

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myocardial infarction is the cardiac disease with the most severe social and economic consequences as it affects more than 6.5 million people in Europe and more than 5 million in the USA [1].

High mortality associated with acute myocardial infarction (AMI) is due to the inability of the heart to replenish the pool of cardiomyocytes massively depleted after the ischemic insult. As a consequence, dead myocardium is replaced by a fibrotic scar, which is the cause of the development of heart failure and cardiac arrhythmias [2,3].

Until now, rebuilding damaged heart has remained a major challenge in regenerative medicine. Although pharmacological and surgical interventions, including coronary revascularization (coronary artery bypass grafting or coronary angioplasty with or without stenting) and multisite pacemakers (in which the left and right ventricles are stimulated at the same time by a pacemaker implanted in the right ventricle and another implanted in the left ventricle), have been successful in reducing the progression of myocardial fibrosis [4,5], these approaches are inefficient in promoting regeneration of the damaged heart. Consequently, they are only able to prolong survival of many patients for the short term. When patients become refractory to optimal current standard therapies, heart transplantation is considered as a last therapeutic option. Nevertheless, only a restricted number of subjects can benefit from such treatment due to the lack of sufficient heart donors, the transplant complications, and the excessive economic costs. In order to face up to these current therapeutic limits, it appears of critical importance to develop novel alternative therapies to achieve widespread regeneration of dead myocardium. In this regard, cell-based therapies, and in particular those based on the use of MSCs, have appeared as one of the most promising approaches to heal heart damage.

### 21.2.2 Mesenchymal stromal cells: a promising tool to treat the effects of myocardial infarction

Recently, cell-based treatment strategies have been introduced as an alternative to rebuild damaged heart tissue and prevent adverse cardiac remodeling and heart failure by allowing the recolonization of the infarcted area with new viable and contractile cardiomyocytes. During the last few years, many stem or progenitor cell populations from a variety of tissue sources have been tested in experimental or clinical settings for their cardiac regenerative ability (Figure 21.1).

Among cell populations tested are skeletal myoblasts, fetal and neonatal cardiac stromal cells, endothelial progenitor cells, embryonic stem cells (ESCs), and induced pluripotent stem cells (iPSCs), the latter two after their differentiation into cardiomyocytes [6–8]. Although, studies have shown that all these cell populations ameliorated heart function when delivered into animals in which an AMI had been induced, translation toward the clinic was seriously hampered by several hurdles inherent to the type of cells used. For instance, the most serious complication of using skeletal myoblasts in humans with AMI was the risk of inducing cardiac arrhythmias due to the lack of electrophysiologic coupling between transplanted skeletal myoblasts following their differentiation into skeletal myofibers with resident cardiomyocytes. As a consequence, a high incidence of sudden death in recipients of skeletal myoblasts has been reported in clinical trials [9–11].

In contrast to skeletal myoblasts, ESCs as well as fetal or neonatal cardiac stromal cells have been shown to differentiate into functional cardiomyocytes *in vivo* and thus were expected to efficiently replenish the dead myocardium. Nevertheless, the use of these cells in the clinic is compromised by limited tissue supply, ethical concerns, the risk of immune rejection, and the risk of teratoma formation should any undifferentiated ESCs remain in the transplantation of the cardiac-differentiated progeny [12–15]. A teratoma is a tumor with tissue or organ components resembling normal derivatives of more than one germ layer. Teratomas have been reported to contain hair, teeth, bone, and, very rarely, more complex organs or processes such as eyes, torso, hands, or feet. Because they are encapsulated, teratomas are usually benign, although malignant teratomas can occur. The risk of teratoma formation due to transplantation of stem cells is thought restricted to the use of ESCs or iPSCs. Teratomas have not been reported to date as a consequence of the transplantation of MSCs.

In an attempt to overcome the ethical and immunological issues encountered with the use of ESCs, rodent and human fibroblasts have been successfully reprogrammed to a pluripotent state through the introduction of several pluripotency-regulating genes. However, the discovery of such ESC-like cells called iPSCs did not unfortunately resolve the aforementioned risk of teratoma formation with the use of ESCs [16–19]. In fact, teratoma formation is one of the routine tests performed on putative ESCs or iPSCs to demonstrate their pluripotent ability.

In order to avoid the complications inherent in the use of ESCs and iPSCs, scientists have paid particular attention to MSCs, as they seem to fulfill the ethical and clinical criteria required for their administration to humans. MSCs are able to differentiate into a broad range of specialized cell types *in vitro*, including adipocytes, osteoblasts, chondrocytes, and also muscle cells, such as cardiomyocytes [20–24], although the latter finding remains controversial. MSCs have been successfully isolated from many different tissues, including skeletal muscle [25], dental pulp [26], synovial membrane [27], lung [28], umbilical cord blood [29], amniotic fluid [30], placenta, and skin [31], although the most studied remain those derived from bone marrow [32] or adipose tissue [33–35]. Interestingly, these cells can be obtained in clinically relevant numbers, can be expanded in large scale *in vitro*, and can be cultured in simple growth medium conditions [36–38]. Furthermore, MSCs exhibit immune privileged behavior, raising the possibility of using them as universal regenerative cells for allogeneic transplantation, without any need for histocompatibility between the MSC recipient and the MSC donor [39].

Studies performed during the last decade strongly indicate that intramyocardial delivery of MSCs ameliorates heart function

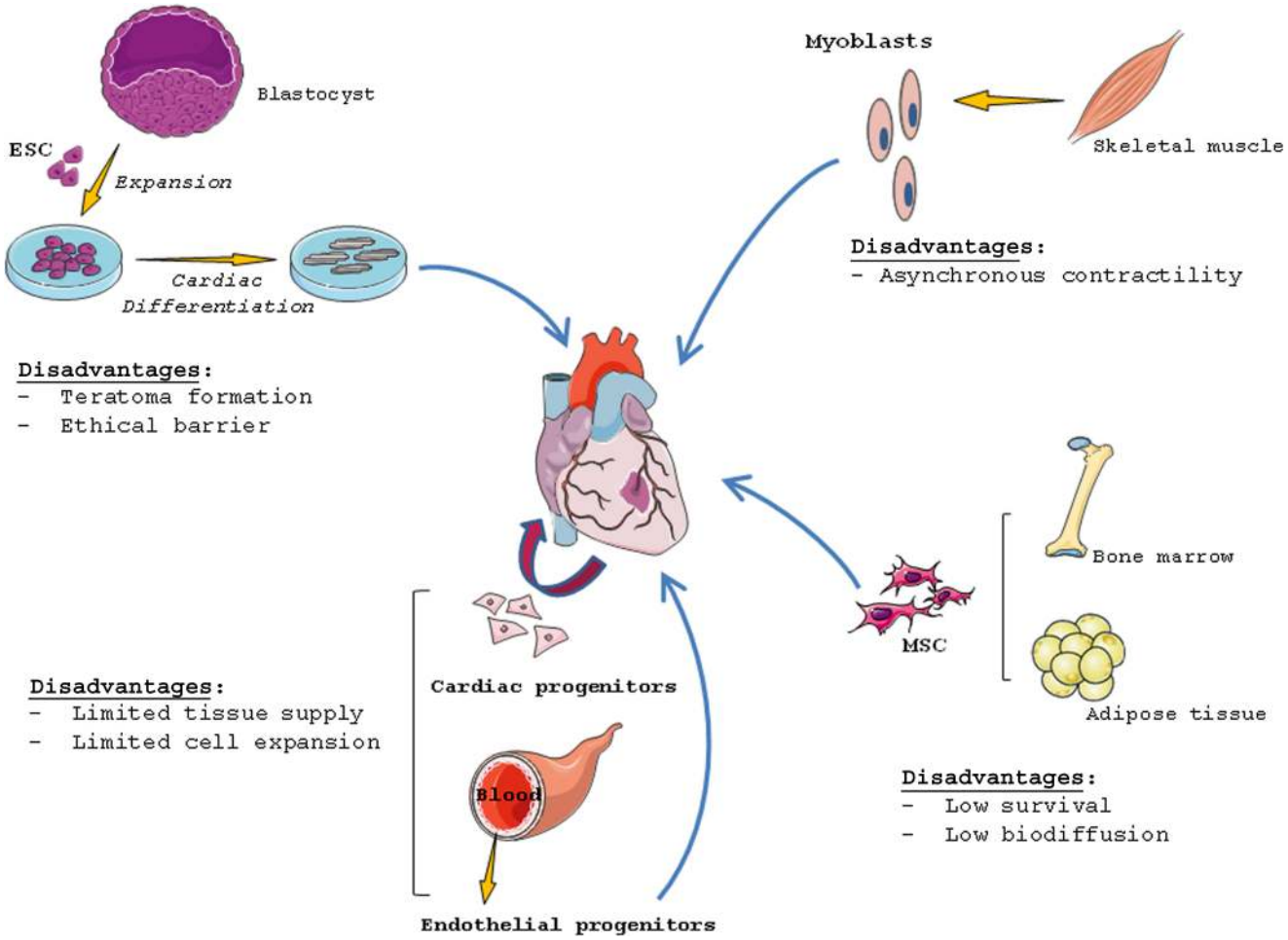



**Figure 21.1** The different kinds of cells used or potentially usable for treatment of cardiac disease. Embryonic stem cells (ESC) must be differentiated into cardiomyocyte-like cells before they can be injected into diseased heart tissue. Skeletal myocytes differentiated into multinucleated myofibers when engrafted into diseased heart. Cardiac progenitors and endothelial progenitors ameliorate heart function primarily by differentiating into cardiomyocytes or endothelial cells respectively. MSCs promote heart repair without significant cardiomyocyte transdifferentiation, but rather by indirect cytoprotective effects. Clinical limitations of the use of each cell type are mentioned in the figure. This figure was produced using Servier Medical Art.

after experimental infarction in different animal models [40–42]. However, such positive findings are less evident in clinical trials, as delivery of MSCs to diseased human hearts did always achieve robust and long-term functional cardiac recovery [43], although, again, this is controversial (see Chapter 45). The disappointing outcomes of some trials underscore the need to develop a greater understanding of the mechanisms underlying the therapeutic benefits of MSCs in the attempt to maximize their effectiveness as a therapy for repair of damaged cardiac tissues.

### 21.2.3 Mechanisms underlying the regenerative effects of mesenchymal stromal cells

Various mechanisms have been proposed for the therapeutic effect of MSC treatment on impaired cardiac function (Figure 21.2).

Transdifferentiation of MSCs into cardiomyocytes or vascular cells [44–46] and permanent cell fusion with resident cardiomyocytes leading to formation of binucleated heterokaryons or hyperploid synkarions [47,48] were initially reported as regenerative modalities employed by MSCs to improve heart function. Nevertheless, the rare occurrence of these processes has cast doubts on their functional impact [47–49].

Another body of published works indicated that MSCs ameliorated cardiac dysfunction by the release of soluble factors. According to these studies, the cardioprotective paracrine action of MSCs is manifested in different ways, including the promotion of new vessel formation, protection of cardiomyocytes against apoptosis, induction of a beneficial extracellular matrix remodeling, and modulation of postinfarction-associated inflammation [50–55]. In addition, some of these studies indicated that MSCs also stimulated myocardial regeneration through the recruitment of bone-marrow-derived stromal cells to the injury site and the activation/differentiation of resident cardiac progenitor cells [50–53].

Recently, a new concept has emerged proposing that the regenerative capacities of infused MSCs might be stimulated

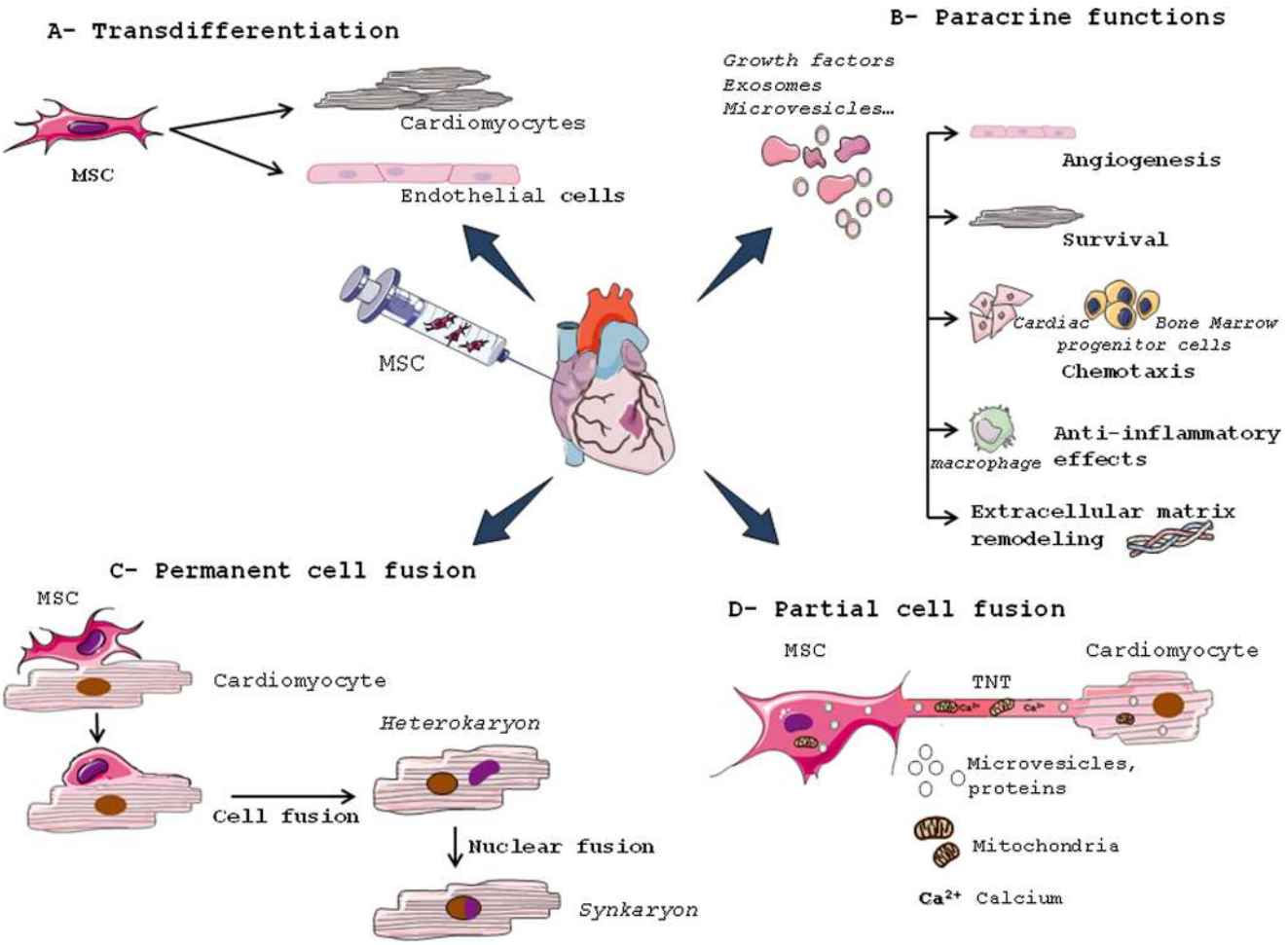



**Figure 21.2** Regenerative mechanisms employed by MSCs to repair damaged myocardium. MSCs can: (A) possibly differentiate into cardiomyocytes and endothelial cells to replace cardiomyocytes lost due to cardiac injury; (B) secrete soluble mediators, including growth factors, cytokines, exosomes, and large microvesicles that exert cytoprotective effects; (C) possibly fuse with cardiomyocytes, leading to the formation of a reprogrammed hybrid heterokaryon or synkaryon; (D) communicate through tunneling nanotube structures (TNTs), allowing intercellular exchanges of a broad range of compounds. This figure was produced using Servier Medical Art.

by the microenvironment in which they engrafted through the release of physiological cues from injured cells in that microenvironment [56]. However, the role of the microenvironment as a key regulator of MSC function is currently poorly understood. Nevertheless, this intriguing issue was retrospectively supported by a series of *in vitro* studies showing that stress cues emitted by damaged cells could be transmitted to MSCs or other progenitor cells by several pathways, including, first, gap junctions, which allow the direct passage of inorganic ions, small cytoplasmic molecules, and genetic material such as microRNAs (miRNAs). The latter have been shown recently to be involved in the differentiation of cardiac stromal cells [57]. A second pathway proposed that soluble factors underwent receptor-mediated transduction into target cells, as illustrated by the release of prostaglandin $E_2$ following activation of caspases 3 and 7 in apoptotic cells, which triggered stromal cell proliferation and tissue regeneration [58]. A third method proposed that integrins mediate interactions of cells with the extracellular matrix, allowing them to sense any stress-induced matrix changes [59–61]. Additionally, stressed cells can communicate with MSCs through the release of extracellular membrane vesicles such as apoptotic bodies, microvesicles, and exosomes [62–64]. These vesicles, which had been initially considered as cell debris, can contain protein, peptides, fragmented DNA, and several classes of mRNA molecules, including miRNAs. These miRNAs represent small noncoding RNAs of approximately 20–22 nucleotides that, upon sequence-specific binding to appropriate mRNAs, repress the translation of the corresponding proteins or induce a subsequent degradation of the miRNA/mRNA [63–65]. Fusion of these vesicles with MSCs can alter their behavior by regulating their proliferation, differentiation [62,64,66], migration [67], and paracrine function [68]. In turn, MSCs can also secrete microvesicles and exosomes to rescue distressed cells or to promote angiogenesis or to regulate inflammation [69,70].

More recently, a novel way of cell-to-cell communication between cells remote from each other has been proposed by which membrane thin channels allow intercellular exchanges of a broad range of cellular components. These latter structures, commonly referred to as tunneling nanotubes (TNTs), as well as their physiological roles will be described in more detail in the following section. Particular attention will be paid as to whether crosstalk mediated by these structures affects MSC fate and the phenotype of the interconnected somatic cells.

## 21.3 Tunneling nanotubes: a universal route of intercellular communication between distant cells

TNTs were initially described by Rustom *et al.* in rat pheochromocytoma PC12 cells as long actin-rich bridges connecting cultured cells [71]. Other cellular projections, such as filopodia and cytonemes, have been previously described. Filopodia (also known as microspikes) are slender cytoplasmic projections that extend beyond the leading edge of lamellipodia in migrating cells. They contain actin filaments cross-linked into bundles by actin-binding proteins, such as fascin and fimbrin. Filopodia form focal adhesions with the substratum, linking it to the cell surface. Many types of migrating cells display filopodia, which are thought to be involved in both sensation of chemotropic cues and resulting changes in directed locomotion.

Cytonemes are thin, cellular projections that are specialized for exchange of signaling proteins between cells. Cytonemes emanate from cells that make signaling proteins, extending directly to cells that receive signaling proteins. Cytonemes also extend directly from cells that receive signaling proteins to cells that make them. A cytoneme is a type of filopodium – a thin, tubular extension of a cell's plasma membrane that has a core composed of tightly bundled, parallel actin filaments. Filopodia can extend more than 100 μm and have been measured as thin as 0.1 μm and as thick as 0.5 μm.

TNTs are novel structures able to ensure membrane continuity between two remote cells allowing intercellular exchanges of a broad range of cellular compounds and without attachment to the substratum [71]. Interestingly, these structures appeared to be highly dynamic as they formed *de novo* within a few minutes and persisted *in vitro* from minutes to several hours [71,72]. They are transient, highly fragile, and sensitive to light exposure, shearing force, and chemical fixation. Since their discovery in 2004, TNTs have been detected in various cells types *in vitro*, including nervous system cells [71,73,74], myeloid cells [75–78], human and murine T cells [79], normal rat kidney cells [80], myogenic skeletal muscle satellite cells [81], retinal pigment epithelial cells [82], osteoclast precursors [83], and several types of malignant cells [84,85]. TNT structures have also been found to connect MSCs with several kinds of somatic cells, including cardiomyocytes [86–89], endothelial cells [90,91], renal tubular cells [92], and vascular smooth muscle cells [93]. They can also connect cancer cells to various types of stromal cells, such as fibroblasts, MSCs, as well as to endothelial cells [94,95] (Figure 21.3).

In contrast to the easy detection of TNT structures in *in vitro* systems, their assessment *in vivo* is extremely difficult because of their fragility, their transient formation, and the lack of known specific markers. Nevertheless, several *in vivo* studies have reported the presence of TNT-like connections in mouse and chick embryos during *blastocyst* formation (the structure formed in the early development of mammals and possessing an inner cell mass that subsequently forms the embryo), *neurulation* (the folding process in vertebrate embryos that includes the transformation of the neural plate into the neural tube) [96–98], *dorsal closure* (the last major morphogenetic process whereby a gaping dorsal epithelial hole – the result of germ band retraction – is sealed closed to cover an otherwise naked extraembryonic amnioserosal sheet [99], and *gastrulation* (a phase early in the embryonic development of most animals, during which the single-layered blastula is reorganized into a trilaminar (three-layered) structure known as the gastrula with the three germ layers known as the ectoderm, mesoderm, and endoderm) [100].

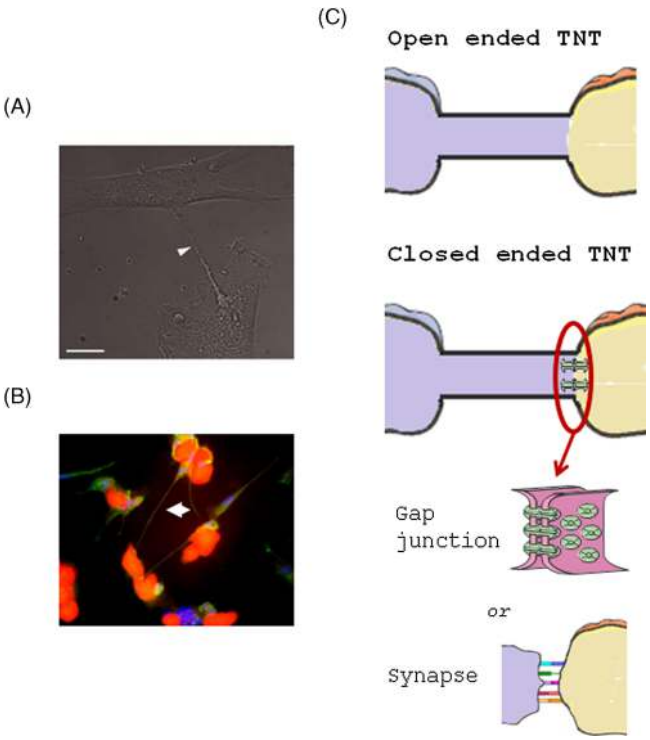



**Figure 21.3** The structural diversity of TNTs. (A) TNT morphology. Live-cell imaging microscopy showing a TNT connection between two MSCs. Scale bar: 20 μm. (B) Cytoskeletal composition of a TNT. Immunocytochemistry showing TNT connections between MSCs and cardiomyocytes (arrowhead) composed of F-actin and microtubules. (C) The two different types of TNT tips. TNT tips can be open-ended or closed-ended. In this latter situation, tips contain additional structures serving cell-to-cell communication such as gap junctions of immunological synapses. This figure was produced using Servier Medical Art.

Although more difficult to observe in adult tissues, TNT-like structures have been detected in mouse cornea [101,102], in rat lung [103], and rabbit kidney nephrons [104]. Furthermore, TNTs have also been found in malignant tissues such as human mesothelioma and lung adenocarcinoma [84].

### 21.3.1 Structural diversity of tunneling nanotubes

Several studies based on live cell imaging microscopy as well as transmission- and scanning-electron microscopy have revealed the structural diversity of TNT bridges with regard to their length, diameter, and cytosketal components. This structural diversity is dependent of the type of cells that are interconnected. Indeed, the average length of TNTs varies from 6–7 μm in adrenal pheochromocytoma PC12 cells [71] or primary neurons and astrocytes [105] to 22 μm in the Jurkat T cell line (originally called JM) established in the late 1970s from the peripheral blood of a 14-year-old boy with T cell leukemia) [79] and cervical-cancer-derived Hela cells [76]. They reach more than 40 μm in human retinal pigment epithelial ARPE-19 cells [82]. The length of TNTs is dynamically regulated, as it increases when connected cells migrate and the distances between them change, in this case exceeding 100 μm. However, TNTs break when the intercellular gap becomes too large [106]. The diameter of TNTs has been shown to vary according to the types of cells connected, ranging from 50–200 nm in PC12 cells [71], 180–380 nm in T cells [79], and exceeding 700 nm in human monocyte-derived macrophages [77], human lung carcinoma A549 cells [107], and rat cardiac H9C2 cardiomyoblasts [108]. Interestingly, these variations in TNT thickness have led to a classification of thick and thin nanotubes [77].

An additional criterion of variation in TNT structures is related to their cytoskeletal composition. All TNTs are composed of an F-actin backbone. Actin is a globular multifunctional protein that forms microfilaments. Actin participates in many important cellular processes, including muscle contraction, cell motility, cell division and cytokinesis, vesicle and organelle movement, cell signaling, and the establishment and maintenance of cell junctions and cell shape. Actin can be present as either a free monomer called G-actin (globular) or as part of a linear polymer microfilament called F-actin (filamentous), both of which are essential for such important cellular functions as the mobility and contraction of cells during cell division.

In some cases, and in particular when TNTs are thick (>0.7 μm), they can also contain microtubules [77]. Finally, another parameter contributing to TNT structural variability is the morphology of the tip that establishes contact with the remote cell (Figure 21.3C). Although TNTs are often considered as open-ended bridges that ensure membrane continuity between the two connected cells through a partial cell-fusion-like process, experiments have revealed the existence of a second kind of TNT that exhibits a closed-ended tip. Interestingly, closed-ended TNTs have been shown to be able to accumulate connexin 43 at one of their ends to form functional gap junctions. Such structures have been found to connect neurons with astrocytes [105] or normal kidney cells themselves [80]. Closed-ended TNTs have also been shown at one of their tips to exhibit protein complexes suggesting the formation of immune-like synapses. For instance, such structures were found to link natural killer (NK) cells to mastocytoma cells. In this study, NK protrusions were found to contain clusters of proteins that are involved in the biogenesis of immune synapses such as the activating receptor NKG2D (an activating receptor found on NK cells and both αβ and γδ $CD8^+$ T cells), its signaling receptor DNAX-activation protein 10, and its ligand major histocompatibility complex (MHC) class I chain-related A. Although the function of TNTs sharing immune synapses remains to be formally determined, we can speculate that such structures ensure the NK cells exert their cytotoxicity on distant target cells [109].

Thus, the series of studies cited in this section reveals the extreme diversity of TNT structures with regard to their cytoskeletal composition, length, and thickness. These variations may reflect an adaptation of these structures to the function that they exert and the kinds of cells with which they connect.

### 21.3.2 Mechanisms and factors involved in tunneling nanotube formation

The mechanisms of TNT formation are not yet fully understood. However, time-lapse imaging has suggested that TNTs can be formed within a few minutes by two modalities. These are the outgrowth of actin-driven protrusions and the dislodgement of connected cells [110] (Figure 21.4). Although these two mechanisms of TNT biogenesis are not mutually exclusive, since they could occur in the same cell type, they seem to be dependent on the mobility of the connected cells. Indeed, mobile cells such as T cells, which can easily physically interact with other cells, are thought to be more adept at forming TNTs mainly by cell dislodgement [79,80], whereas more immobile cell types, such as those of the PC12 cell line, are prone to form TNTs by *de novo* outgrowth and extensions of actin-driven protrusions toward distant cells [72].

#### 21.3.2.1 Formation of tunneling nanotubes by actin-driven protrusions

This process which was initially proposed by Rustom and colleagues in 2004 [71], consists of the ability of either one or both the cells destined to come into contact to induce the outgrowth of tubes containing F-actin (Figure 21.4A). The molecular mechanisms involved in the emergence of TNT protrusions are still unclear. However, these mechanisms probably require the activation of cell division control protein 42 (CDC42), a member of the Rho family of GTPases, to initiate the actin nucleation complex [111], which in turn enables the extension of the protrusion through actin polymerization toward the target cell. After elongation, the tip of the protrusion enters into contact with the targeted cell and fuses with its membrane. This step, which requires energy, has several implications. First, it could involve fusion proteins such as soluble *N*-ethylmaleimide-sensitive factor activating protein receptor proteins [112].

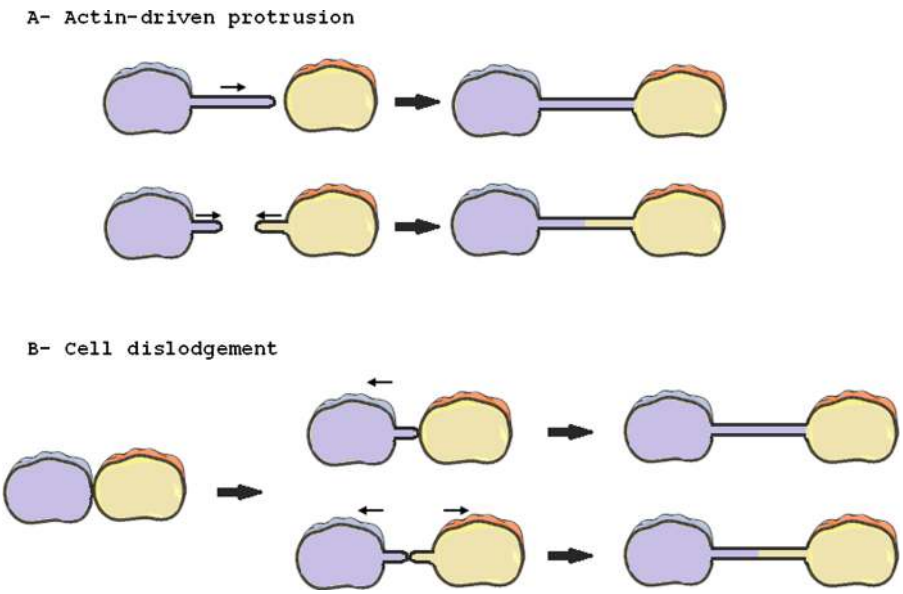



**Figure 21.4** The two modalities of TNT biogenesis. TNTs can be created through the process of (A) actin-driven protrusion or (B) cell dislodgment. This figure was produced using Servier Medical Art.

Second, it could require an appropriate lipid bilayer membrane that could confer a high degree of membrane curvature and lead to the spontaneous membrane fusion of the TNT tip with the target cell [113]. Such a process is often involved in the generation of TNT structures connecting neuronal cells [72,73], kidney cells [71], and probably MSCs with various other cell types, including cardiomyocytes [88,89], endothelial cells [90], vascular smooth muscle cells [93], and renal tubular cells [92]. Further studies are required to identify the molecular protagonists triggering TNT emergence, elongation, and fusion.

#### 21.3.2.2 Formation of tunneling nanotubes by cell dislodgement

According to the second mechanism postulated, TNTs arise from cells initially in close contact with their target cell type and with whose membranes they have fused. Subsequently, when these cells migrate in opposite directions, thin thread membrane tethers are drawn out, leading to the formation of TNTs that could belong entirely to one cell or to both cells [71,110] (Figure 21.4B). The mechanism of cell dislodgement leads to the creation of closed-ended TNTs that can exhibit at one of their tips either gap junctions [80,105], synapses [109], or receptors [79]. TNTs derived from a cell dislodgement mechanism have been shown to connect with a broad range of cells, including immune cells [77–79], normal kidney cells, neural crest cells, and primary human umbilical vein endothelial cells (HUVECs) [80]. Whether MSCs communicate through TNT bridges arising from a cell dislodgement process is still unknown. Furthermore, the cell dislodgement mechanism remains poorly characterized. In particular, it remains to be determined whether this process requires adhesion molecules or fusogenic proteins and to identify the signaling cues triggering the migration between the two cells involved in the TNT process.

#### 21.3.2.3 Factors involved in tunneling nanotube formation and regulation

Characterizing the signals and the molecular protagonists that induce TNT formation will be useful for understanding the physiological roles of TNT connections. There is emerging evidence indicating that stress signals and inflammatory cues are important regulatory factors of TNT biogenesis.

Indeed, cellular stresses induced by hydrogen peroxide or serum starvation have been shown to increase the number of TNTs in different cell types, including astrocytes and neurons [74]. In addition, data from our own laboratory have shown that priming of MSCs with inflammatory cytokines such as interferon (IFN)-γ and tumor necrosis factor (TNF)-α heighten the number of TNT connections between human MSCs and distressed cardiomyocytes [89]. Consistent with our *in vitro* studies, cues from inflammatory tissue have been reported as stimulating the formation of TNTs *in vivo* between dendritic cells located in the mouse cornea [101]. Since acute inflammation is commonly associated with tissue injury, we can speculate that pro-inflammatory signals are received by MSCs as harmful signals. As a consequence, these cells might create TNT extensions either to rescue neighboring stressed somatic cells or to counteract side effects from excessive inflammatory processes through direct interaction with immune cells such as macrophages or T lymphocytes. Further studies are required to determine whether inflammation regulates TNT-mediated cell-to-cell communications.

Another important question is related to the type of cells involved, specifically whether it is stressed or unstressed cells that initiate TNT elongation. Although there is emerging evidence that stressed cells release the upstream signals triggering TNT biogenesis, it remains to be determined if these signals attract filopodia-like protrusions from unstressed cells to stressed ones or if stresssed cells are the initiators of TNT protrusions. The

limited experimental data currently available are quite discrepant. It has been shown in co-cultures containing neurons or astrocytes damaged by treatment with hydrogen peroxide or by serum starvation that stressed cells develop TNT towards safe cells as their targets [74]. Conversely, another study was performed with endothelial progenitor cells and endothelial HUVECs previously exposed to glycated collagen I in order to induce lysosomal dysfunction and premature senescence. In this situation, progenitor cells initiated outgrowth of TNT bridges toward damaged cells [91].

These apparent discrepancies could be explained by differences in the type of initiator and receptor cells used to carry out the experiments. In addition, the kind of injury applied to initiator cells, which quantitatively and qualitatively influences the release of harmful cues, might also be an important factor involved in the decision of the cell source from which TNT protrusions must be initiated.

In addition to stress signals and inflammation, several proteins and signaling pathways have been reported to be involved in TNT formation. Wang *et al.* [74] reported that activation of p53 protein, which can induce apoptosis, or the EGF receptor through the stimulation of the Akt/PI3K/mammalian target of rapamycin pathway, was responsible for open-ended TNT formation in cultured neurons and astrocytes following stress induced by treatment with hydrogen peroxide.

Further, mSec and the Ral/exocyst complex, which is a filopodium-dependent mechanism, have been shown to be involved in the formation of TNTs in macrophages and cervical cancer Hela cells [76] as well as neurons and astrocytes. (The exocyst is an octameric protein complex involved in vesicle trafficking, specifically the tethering and spatial targeting of post-Golgi vesicles to the plasma membrane prior to vesicle fusion. It is implicated in a number of cell processes, including exocytosis and also cell migration and growth.)

Interestingly, Wang *et al.* have suggested that mSec effects are probably under the control of activated p53 [74]. More recently, Schiller and colleagues have provided evidence of the critical role of the leukocyte-specific transcript 1 protein in promoting the assembly of the multimolecular complex (mSec, the small GTPase RalA, and the exocyst complex) that together orchestrate the formation of functional TNTs [114].

Finally, activation of FAS signaling in immune cells has also been shown to increase the number of TNTs by activating Rho GTPases and CDC42 (a member of Rho small GTPase family) [115]. Fas forms the death-inducing signaling complex upon ligand binding.

Overall, the studies carried out to date have revealed the diversity of TNT structures and formations. This diversity likely results from several parameters: first, the nature of the stress signals emitted by the damaged cells, which in turn may govern activation of the appropriate signaling pathways for the initiation of TNT formation; second, the physiological role that TNTs mediate; and third, the type of cells involved.

## 21.3.3 The diversity of compounds transferred by tunneling nanotubes and their physiological relevance

### 21.3.3.1 The characteristic features of tunneling nanotube transfers

The multifaceted functions of TNT channels are illustrated by the broad range of compounds that TNTs transfer between connected cells. These compounds include organelles, proteins and vesicles containing genetic material, ions and pathogens, as well as electrical signals. Besides the heterogeneity of the cargoes transported by TNTs, it is interesting to note that such a transfer is not a single diffusion process but rather is highly regulated, since it can be either unidirectional or bidirectional according to the compounds that are conveyed. Although the specific signals and mechanisms responsible for directing traffic remain largely unknown, this process seems to be dependent on the structural composition of TNTs: bidirectional transfers have been reported as being associated with TNTs containing both actin and microtubules [77,88,115,116], while unidirectional ones have been associated with TNTs containing actin only [71,75,117]. Furthermore, several studies have shown that the direction of these exchanges is also influenced by the physiological states of the two connected cells. As an example, lysosomal exchanges from endothelial progenitor cells to HUVECs have been shown to be selectively augmented when HUVECs exhibited lysosomal dysfunction and premature senescence following glycated (nonenzymic glycosylation) collagen treatment [91]. Such a phenomenon appears to be devoted to restoring the pool of functional lysosomes in defective cells. Likewise, unpublished data from our laboratory indicate that transfer of mitochondria from stromal cells to somatic cells, such as fibroblasts, cardiomyocytes, and endothelial cells, is much more important when somatic cells have been exposed to $H_2O_2$-mediated oxidative stress prior to the co-culture, which leads to mitochondrial dysfunction. In addition, Plotnikov *et al.* described that intercellular exchanges between human multipotent stromal cells and rat renal tubular cells were more prominent toward stromal cells, thus favoring their differentiation into kidney tubular cells [92]. According to these studies, movement of cargoes along TNT bridges appears to be finely regulated, in agreement with expectations such as the rescue of injured cells, progenitor fate changes, or the immunological lysis of infected cells.

Another important point is that transfer mediated by TNTs appears to be an active process dependent on cytoskeletal actin and requiring ATP consumption. The importance of cytoskeletal components and ATP supply has been shown for the transfer of organelles and vesicles [72,73,77,118].

### 21.3.3.2 Signal transfer

#### *21.3.3.2.1 Transfer of calcium ions and electrical currents*

TNTs have been shown to propagate calcium signals between remote cells and thus constitute an alternative to gap junctions, which only allow transfer of calcium fluxes between cells in close proximity to each other. This kind of transfer has been

found in many cells types able to make connections between each other.

Initially, propagation of calcium fluxes through TNTs was observed between myeloid cells following stimulation with *Escherichia coli* supernatants or mechanical stress [78]. This study indicated for the first time that calcium transported along TNTs might be involved in initiating and amplifying pathogen signals in connected sentinel cells responsible for triggering immune responses, such as dendritic cells [78]. TNT-mediated calcium fluxes have also been observed between neurons and astrocytes [105], human retinal pigment epithelial cells [82], normal rat kidney cells [80], as well as the HEK293 and neuroblastoma SH-SY5Y cell lines [119].

Propagation of intercellular calcium signaling through TNT channels is probably important in contributing to several physiological processes, including the regulation of the antibacterial immune response [78] as mentioned earlier, the coordination of the migration and differentiation of neural progenitor cells during embryonic development [120], and the stimulation of wound healing processes triggered by membrane depolarization at the leading edge of the wound. Such depolarization leads to the activation of several downstream signaling pathways involving protein kinase A and PI3kinase, which in turn lead to synchronization of actin remodeling and repair [121–124].

According to the type of cells involved, TNT-mediated transfer of calcium can be conferred either by passive diffusion [78] or, in contrast, through an active process requiring ATP and specialized protein complexes such as IP3 receptors, which may actively propagate calcium ion $Ca^{2+}$ through a $Ca^{2+}$-induced calcium release mechanism [119], or through gap junctions that contribute to the spread of electrical currents [80,82,105].

#### *21.3.3.2.2 Transfer of death signaling*

Besides ensuring $Ca^{2+}$ signaling and electrical coupling, TNTs can also serve as conduits for the dissemination of death signals. This was illustrated by the study of Arkwright *et al.*, who reported that stimulation of FAS signaling, which is important for rapid cell death, significantly increased the number of TNT connections in T lymphocytes [115]. This process may significantly contribute to the peripheral deletion of autoreactive lymphocytes by ensuring the rapid propagation of apoptosis signals within themselves. The involvement of TNT structures in controlling autoimmunity has been strengthened by *in vivo* observations in patients with the autoimmune lympho-proliferative syndrome (ALPS). This is a form of lymphoproliferative disorder that affects lymphocyte apoptosis. It is a disorder of Ras signaling – a RASopathy. Normally, after an infection, the immune system downregulates by increasing Fas expression on activated B and T lymphocytes and Fas ligand (CD95L) on activated T lymphocytes. Fas and Fas ligand interact to trigger the caspase cascade, leading to cell apoptosis. Patients with ALPS have a defective apoptotic pathway of T lymphocytes [125] and mutations of the Fas receptor [126,127]; T lymphocytes are unable to form TNT connections [115]. This syndrome leads to chronic nonmalignant lymphoproliferation, autoimmune disease, and secondary cancers.

A second example of the transfer of death signals was provided by the work of Chaveau *et al.* showing that closed-ended TNT structures with an immune synapse at one of their tips enabled NK cells (lymphocytes involved in the elimination of infected or malignant cells) to eradicate target cells [109]. The regulatory role that TNTs exert on NK-mediated cytotoxicity is strengthened by the fact that inflammatory cytokines such as interleukin (IL)-2, -12, -15, and -18 increase the number of TNT protrusions generated by NK cells [109].

### 21.3.3.3 Transfer of organelles, proteins, and genetic material

#### *21.3.3.3.1 Transfer of organelles*

Several *in vitro* studies have reported that TNT channels promote the conveyance of various organelles, such as mitochondria and lysosomes. Interestingly, organelle transfer along TNTs is the best example in which the direction of this phenomenon is clearly dependent of the physiological state of connected cells. Transfer of these organelles is bidirectional between healthy cells, while it is usually unidirectional when injured cells are connected to normal stem or progenitor cells. Organelle transfer occurs predominantly from the healthy to the abnormal cells. Several studies in the literature indicate that transfer of organelles and particularly mitochondria are aimed at rescuing dying cells. Mitochondria exert critical functions in maintaining the life, or regulating the death, of cells. Mitochondria are commonly considered as the powerhouse of living cells as they produce more than 90% of energy, in the form of ATP through the oxidation of pyruvate in the Krebs cycle, and oxidative phosphorylation. In addition, mitochondria are key sensors of cellular stress and trigger programmed cell death by rupture of electron transport and energy metabolism, by releasing cytochrome c, by activating caspase proteins, and by producing reactive oxygen species [128]. Following exposure to injury, damaged cells try to eliminate their abnormal mitochondria through sequestration, followed by their digestion into autophagolysosomes in the process known as autophagy. Consequently, uptake of functional mitochondria from safe (stem) cells might be a valuable alternative for diseased cells to replenish their pool of organelles and thus preserve their energy metabolism. Occurrence of mitochondrial transfer by TNT structures has been described *in vitro* between stromal cells and various damaged cells, including cardiomyocytes, endothelial cells, and bronchial epithelial cells [88–90,103]. Transfer of mitochondria to stressed cardiomyocytes will be discussed in Section 21.4. Whether TNTs confer protection against apoptosis through mitochondria conveyance has been well illustrated by the recent study of Liu *et al.*, which showed that TNT-mediated mitochondria transferred from MSCs to endothelial cells, previously injured by oxygen or glucose deprivation, improved their survival by preserving their aerobic respiration and ATP production [90]. Strikingly, no protection against apoptosis occurred when injured endothelial cells were co-cultured with

MSCs that had been depleted of functional mitochondria, thus confirming the pro-survival role of stromal-cell-delivered mitochondria [90]. The benefit of stromal-cell-derived mitochondria delivery through TNTs has been confirmed *in vitro* as well as *in vivo* in cigarette-smoke-exposed epithelial airways [103].

However, although transfer of mitochondria is usually beneficial for healing damaged cells, this process may sometimes be deleterious, by, for instance, favoring tumor development. Indeed, increasing mitochondrial activity in cancer cells by the means of TNT channels has been recently reported to increase resistance to cytotoxic drugs and to increase survival of malignant cells [95]. Finally, and in combination with uptake of exogenous mitochondria, TNT-mediated supply of lysosomes can contribute to prolonged survival of diseased cells by helping eliminate dying mitochondria causing deleterious effects by fusing with autophagosomes to form autophagolysosomes [91,129].

#### *21.3.3.3.2 Transfer of protein complexes and genetic material*

TNT channels ensure exchange of proteins and genetic material that allow activation of the recipient cells over long distances. Several kinds of proteins have been shown to travel along TNT bridges. These include the multidrug resistance p-glycoprotein found in breast cancer cells, which increases their chemoresistance [85], and MHC class I complex bound to antigen between dendritic cells, which optimizes antigen presentation to, and the cytotoxic response of, alloreactive T cells [130]. Additionally, genetic material such as miRNA and DNA can be transferred by TNTs from one cell to another in order to reprogram the recipient cell. Transfer of miRNA has been well documented in *in vitro* cancer models, especially from ovarian carcinoma or osteogenic sarcoma tumor cells to stromal cells. Occurrence of this phenomenon could modify gene expression of stromal cells and favor malignant progression [94]. TNT structures have been shown to increase chemoresistance of cancer cells through genetic exchanges between themselves [131].

#### 21.3.3.4 The role of tunneling nanotubes in the spread of pathogens

It has been shown that different pathogens actively hijack TNTs like Trojan horses to be transported along their membranes or inside them, leading to the spread of infections. This transfer is often preceded by the activation of TNT formation, thus optimizing pathogen dissemination. This has been observed for exchanges of HIV particles between macrophages [75]. TNT structures also serve as conduits to allow bacteria to pass from one cell to another without contacting the extracellular environment. For instance, several species of bacteria – including *Mycobacterium bovis*, which causes tuberculosis in cattle and from which the antituberculosis vaccine BCG was derived – have been reported to "surf" TNTs between macrophages prior to being internalized by endocytosis [77].

Apart from viruses and bacteria, Gousset and colleagues provided evidence that TNT channels enable prion spreading between neurons themselves or between bone marrow-derived dendritic cells and primary neurons [73,132]. These findings strongly suggest that TNTs play an important role *in vivo* in the spreading of prions within both the central and peripheral nervous systems [133]. Interestingly, the same authors showed that prion particles travel in early endosomes and lysosomes but are preferentially enriched in the endosomal recycling compartment, thus revealing the efficiency of TNTs in permitting the passage of prions to one infected cell to an uninfected one [73].

## 21.4 Tunneling nanotubes: a novel cell-to cell communication pathway improving the cardiac regenerative properties of mesenchymal stromal cells

### 21.4.1 Evidence of tunneling-nanotube-mediated communications between stromal cells and cardiomyocytes

MSCs have been found to communicate with several types of specialized cells through TNTs, including cardiomyocytes and endothelial cells (Figure 21.5).

The structure of these channels in this setting has not been characterized in depth. No data are currently available on the signals and mechanisms involved in their formations. However,

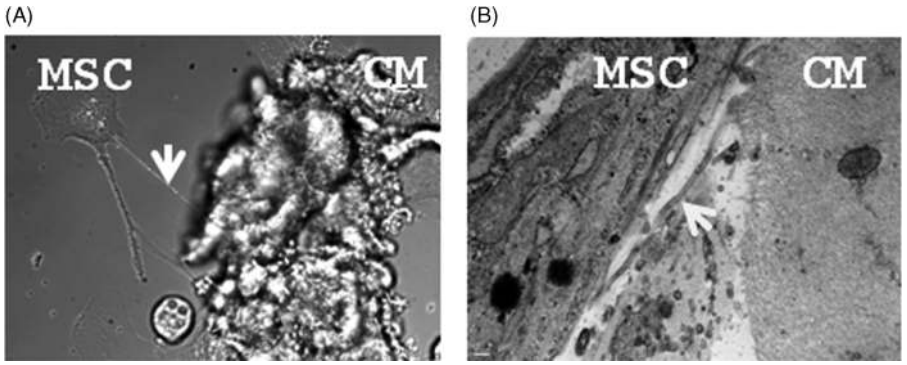



**Figure 21.5** Morphology of TNT structures connecting stromal cells to cardiac cells: (A) live cell microscopy; (B) transmission electron microscopy. Arrow designates TNT. MSC: mesenchymal stromal cell; CM: cardiomyocyte.

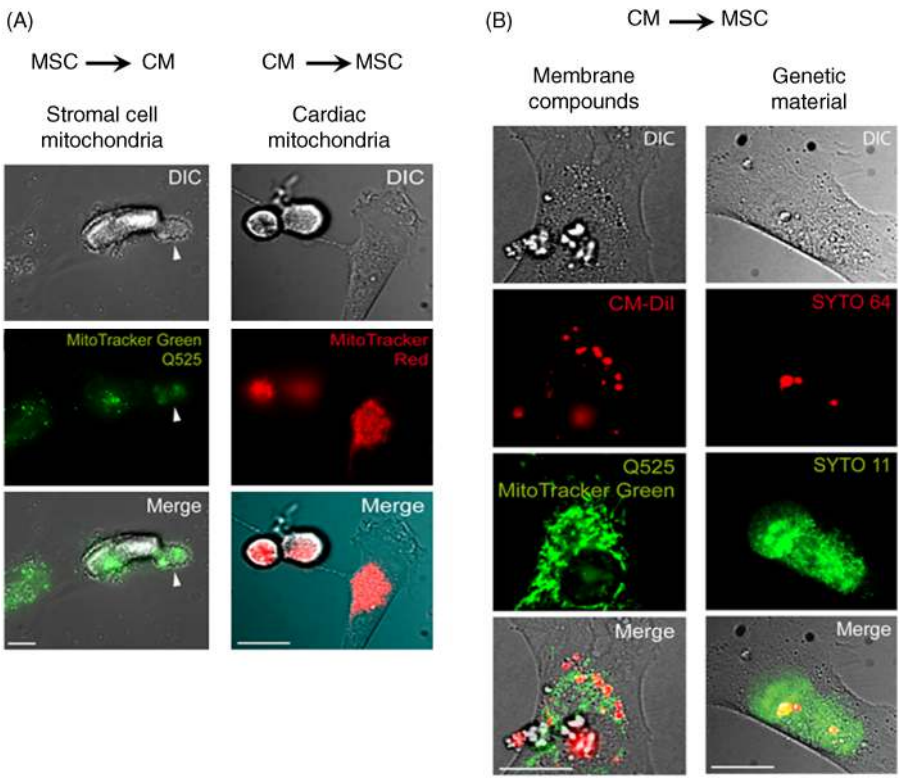



**Figure 21.6** Diversity of intercellular exchanges between MSCs and cardiomyocytes (CMs). (A) Bidirectional exchanges of mitochondria between MSCs and CMs. Left panel: detection of stromal cell mitochondria (green staining using MitoTracker Green FM in cardiomyocytes (white arrowhead). Right panel: detection of cardiac mitochondria (red staining, using Mitotracker red FM in MSCs (white arrowhead). Scale bars: 20 µm. (B) Selective unidirectional exchanges from CMs to MSCs. Left panel: live cell imaging showing that MSCs stained with quantum dot Q525 and MitoTracker Green FM dyes (green signal) contain cardiomyocyte cytoplasmic macromolecules stained previously with CM-DiI dye (red signal). Right panel: live cell imaging showing an MSC with its nucleus stained with SYTO11 dye (green signal) and cardiac DNA stained with SYTO 64 dye (red signal). Scale bars: 20 µm. (See color plate section.)

we have found that these connections are mainly composed of F-actin and microtubules and are open-ended structures, leading to the exchange of several cellular compounds, including cell membranes, DNA material, and organelles, including mitochondria and lysosomes. We have shown that transfer of cytoplasmic and genetic material was unidirectional from stressed cardiomyocytes to MSCs, while transfer of mitochondria appeared to occur in both directions (Figure 21.6).

Interestingly, we also observed that cellular components of cardiomyocytes can be exchanged between MSCs themselves to seemingly accelerate the propagation of signaling cues coming from the cardiomyocytes to the MSC population (Figure 21.7).

Although the exact role of the traffic of most of the compounds between stromal cells and cardiac cells remains elusive, great attention is currently being paid to the physiological impact of cell-to-cell transfer of mitochondria. Several questions must be addressed to clearly understand the mechanisms involved in the formation of TNTs and to fully determine their structures and functions. In particular, it remains to be determined whether MSCs and cardiac cells can communicate through closed-ended bridges containing connexins in one of their tips, whether spreading of $Ca^{2+}$ fluxes and electrical wiring occur along these connections, and, finally, from which cell population (MSCs versus cardiomyocytes) do the TNTs originate. Despite the need to improve knowledge of TNT crosstalk between stromal cells and cardiac cells, current data in this field already indicate that this way of cell-to-cell communication might be critical to the activation of regenerative functions of MSCs, the main goal of which is the rescue of damaged cells after tissue injury. The following subsections provide an overview of recent data obtained in our laboratory describing whether TNT-mediated crosstalk between MSCs and distressed cardiomyocytes affects the phenotype of these two connected cells and thus contributes to cardiac regeneration.

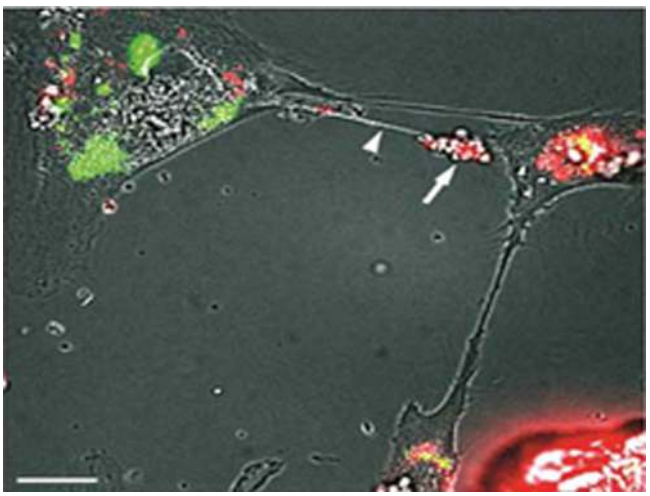


**Figure 21.7** TNT-mediated intercellular exchanges of cardiac compounds between MSCs themselves. Live cell imaging microscopy showing transfer of cardiac cytoplasm (labeled in red with CM-DiI dye, arrow) through a nanotube (arrowhead) connecting two MSC cells (stained with Q525, green signal). Scale bars: 20 µm. (See color plate section.)

### 21.4.2 Tunneling nanotube cell-to-cell communication with mesenchymal stromal cells rejuvenates distressed cardiomyocytes through a progenitor-like state

The question of whether TNT cell-to-cell communication with MSCs might affect the phenotype of adult mammalian cardiomyocytes in a distressed state was addressed in depth in the study of Acquistapace *et al.* [88]. We will summarize here the major findings of this work, which to our knowledge remain unique in this field. This study was conducted in a species-mismatch co-culture system in which human MSCs from adipose tissue or bone marrow were mixed with mouse adult cardiomyocytes, more than 90% of which were dying as assessed by flow cytometry analysis of annexin V/7ADD staining. First, characterization of the xenogeneic (heterologous) cell fusion processes, which had been reported earlier to occur *in vivo* between engrafted stromal cells and resident cardiomyocytes, was explored [48,134–137]. For this, the classical Cre/lox P strategy was used, which allows LacZ gene expression only in fused cells by co-cultivation with Cre-expressing human MSCs and cardiomyocytes from transgenic Rosa26R reporter mice.

Cre–Lox recombination is known as a site-specific recombinase technology, and is widely used to carry out deletions, insertions, translocations, and inversions at specific sites in the DNA of cells. It allows the DNA modification to be targeted to a specific cell type or be triggered by a specific external stimulus. The system consists of a single enzyme, Cre recombinase, that recombines a pair of short target sequences called the Lox sequences. This system can be implemented without inserting any extra supporting proteins or sequences. The Cre enzyme and the original Lox site called the Lox P sequence are derived from bacteriophage P1. Placing Lox sequences appropriately allows genes to be activated, repressed, or exchanged for other genes. The activity of the Cre enzyme can be controlled so that it is expressed in a particular cell type or triggered by an external stimulus like a chemical signal or a heat shock. X-gal is an analog of lactose, and therefore may be hydrolyzed by the β-galactosidase enzyme, which cleaves the β-glycosidic bond in D-lactose. X-gal, when cleaved by β-galactosidase, yields galactose and 5-bromo-4-chloro-3-hydroxyindole. The latter then spontaneously dimerizes and is oxidized into 5,5′-dibromo-4,4′-dichloro-indigo, an intensely blue product that is insoluble. Since X-gal itself is colorless, the presence of blue-colored product can therefore be used as a test for the presence of active β-galactosidase. This easy identification of an active enzyme allows the gene for β-galactosidase (the *lacZ* gene) to be used as a reporter gene in various applications.

After 2 days of co-culture, LACZ-positive hybrid cells were observed. These hybrid cells were organized into colonies and exhibited a small rounded morphology that differed from that of both MSCs and cardiomyocytes (Figure 21.8).

Interestingly, analysis of cell fusion mechanisms leading to the generation of these hybrid cells ruled out a prominent role for permanent cell/nuclear fusion processes since binucleated heterokaryons or synkaryons containing hybrid nuclei were never

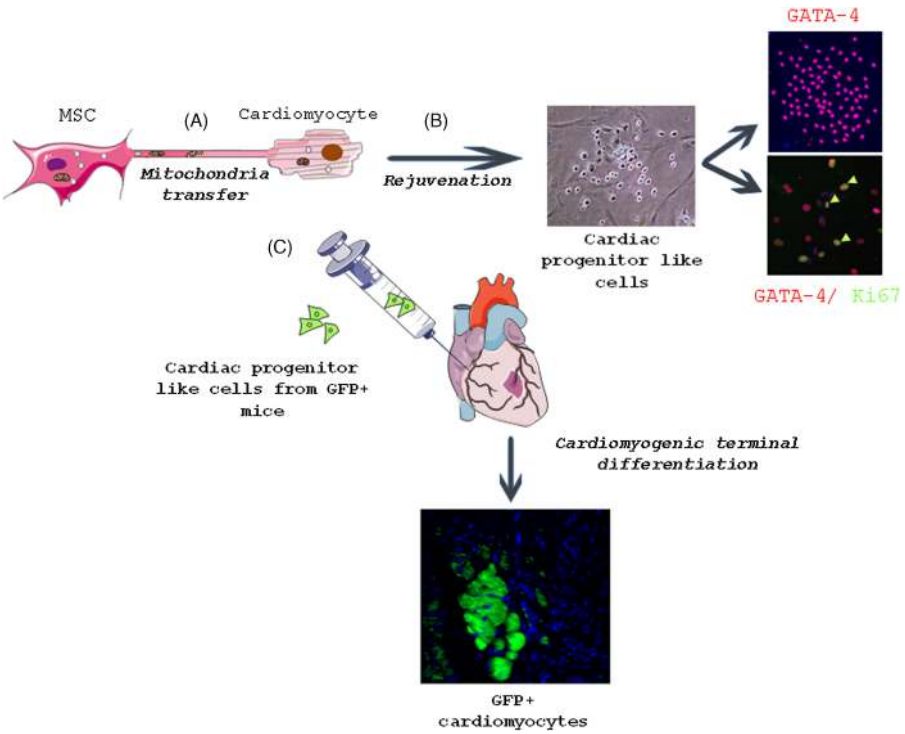



**Figure 21.8** TNT cell-to-cell communication allows MSCs to reprogram dying cardiomyocytes back to a progenitor-like state. (A) MSCs transfer mitochondria along TNTs to a dying cardiomyocyte to (B) prolong its survival and its rejuvenation into progenitor-like cells. These cardiac progenitors organized in colonies express the early cardiac marker GATA-4 and the proliferative marker Ki67. (C) Engraftment of these progenitors (GFP-positive) into mouse infarcted heart muscle leads to their differentiation into functional cardiomyocytes (green fluorescence). This figure was produced using Servier Medical Art. (See color plate section.)

detected by fluorescence *in situ* hybridization experiments. (A heterokaryon is a multinucleate cell that contains genetically different nuclei. Synkaryons are cells where the nuclei from two different parent cells have fused.)

Rather, the authors demonstrated that hybrid cells originated from partial cell fusion involving TNT structures, their generation being impeded in the presence of inhibitors of TNT formation such as nocodazole or latrunculin A. Strikingly, the molecular characterization of these hybrid cells by various methods, including real-time polymerase chain reaction assays using species-specific primers and immunocytochemistry, revealed that these cells displayed a cardiac progenitor-like phenotype. Similar to cardiac progenitors residing in the heart, hybrid cells were able to enter the cell cycle and proliferate, re-expressing early cardiac markers such as GATA-4, Nkx2.5, and MEF-2C. In addition, they had repressed expression of late cardiomyocyte differentiation markers such as desmin, cardiac troponin, and α-sarcomeric actinin, which led to the disappearance of their striated sarcomeric structures and thus the loss of contractile activity. Cytogenetic analysis demonstrated that nuclei of hybrid cardiac progenitor-like cells contained only mouse DNA material, supporting the view that these cells did not derive from transdifferentiation of MSCs into cells of cardiac lineage, but rather from fate changes of adult fully differentiated cardiomyocytes. Taken in concert, these findings indicate that MSCs can mediate the reprogramming of mouse cardiomyocytes back to a progenitor-like state through TNT bridging.

Importantly, additional findings from this study strongly suggest that the capacity to rejuvenate post-mitotic cardiomyocytes toward a more primitive state is restricted to stromal cells, since occurrence of this phenomenon was observed with multipotent stromal cells from adipose tissue and bone marrow as well as pluripotent embryonic cells (unpublished data), but not with human fibroblasts despite the fact that these cells established xenogeneic TNT connections with cardiomyocytes.

The fact that MSCs have the capacity to rejuvenate distressed cells through TNT structures raises the important questions on whether this phenomenon occurs *in vivo* and whether it contributes to healing of cardiac damage. Despite the rare frequency of reprogramming events detected in our *in vitro* experimental setting (due probably to the fact that primary myocytes were also damaged and died prior to being reprogrammed), several *in vivo* observations supported the fact that dedifferentiation and reprogramming of adult cardiomyocytes takes place in mammalian hearts under pathological conditions. Such events have been observed in infarcted myocardium as well as in hibernating myocardium and cardiomyopathy [138,139]. Hibernating myocardium is a state in which some segments of the myocardium exhibit abnormalities of contractile function and usually occurs in the setting of chronic myocardial ischemia.

Such a phenomenon might enable cardiomyocytes to survive adverse conditions such as hypoxia and ischemia [140,141]. More immature progenitor cells are able to adapt their metabolism to reduced oxygen supply by producing ATP by glycolysis (rather than by oxidative phosphorylation, as is the case in fully differentiated cardiomyocytes) and by reducing their energy consumption by the loss of their contractile apparatus. In addition, the dedifferentiation/reprogramming process might also facilitate tissue regeneration by providing a pool of progenitor cells able to proliferate and then to differentiate in order to compensate for the loss of contractile cardiomyocytes destroyed during the injury to the heart. The phenomenon of dedifferentiation/reprogramming, which remains poorly characterized in mammals, has been observed in the regeneration of several organs, including the heart in zebrafish and amphibians [142,143]. A similar phenomenon occurs frequently in plants, which allows fully differentiated, nondividing mesophyll cells to generate pluripotent protoplast cells that are able to form entire plants [144]. Likewise, unpublished data from our laboratory suggest that cardiac progenitor cells generated through TNT cell-to-cell communications between human MSCs and dying murine cardiomyocytes can contribute to healing of cardiac damage as they were shown to generate functional contractile cardiomyocytes when engrafted into the site of myocardial infarction in the mouse (unpublished data).

It is important to note that the cardiomyocyte reprogramming/rejuvenation process mediated by MSCs is finely regulated by stress cues from the microenvironment. This phenomenon occurred *in vitro* when MSCs were co-cultured with damaged cardiomyocytes but not with healthy nenonatal cardiomyocytes [86,87]. However, despite data suggesting that the occurrence of cardiomyocyte reprogramming/dedifferentiation in diseased heart tissue may be beneficial for tissue repair, it remains to be determined *in vivo* whether engrafted MSCs or resident progenitors participate in these events, and whether TNT structures contribute to these effects.

Other important issues concern the mechanisms by which MSCs mediate the rejuvenation of cardiomyocytes and, in particular, to determine the nature of the compounds transported along TNTs that are required for cardiomyocyte reprogramming. Although there may be multiple such compounds, we have focused primarily on the role of mitochondria transferred from stromal cells to cardiac cells because, astonishingly, we detected stromal-cell-derived mitochondria in the reprogrammed cardiomyocytes. To determine the importance of stromal-cell-derived mitochondria in the cardiomyocyte reprogramming process, stromal cells were depleted of mitochondrial DNA by long-term exposure to low doses of bromide ethidium in the presence of uridine and pyruvate, to allow a metabolic switch from oxidative phosphorylation to glycolysis. In these conditions, cardiomyocyte reprogramming did not occur, demonstrating the critical role of these organelles in this process. Whether transferred stromal-cell-derived mitochondria participate in cardiomyocyte reprogramming remains unclear. Such a transfer may enable

prolongation of cardiomyocytes survival since it has been reported in co-culture systems performed with endothelial or epithelial cells [90,103]. This hypothesis is supported by *in vitro* and *in vivo* observations showing that transfer of stromal-cell-derived mitochondria protect cardiomyocytes against apoptosis and is beneficial for functional cardiac recovery. A beneficial role of mitochondria in ischemic heart muscle was also recently confirmed by Masuzawa *et al.*, who showed that injection of exogenous mitochondria into ischemic mouse heart tissue conferred protection against the deleterious effects of ischemia–reperfusion injury [145].

It is important to determine if such mitochondrial transfer have only a pro-survival function or if they also play a direct role in the somatic reprogramming of cardiomyocytes by contributing to the remodeling of their transcriptional machinery through mitochondria-to-nucleus retrograde signaling [146].

Another important question concerns the molecular mechanisms that regulate the fluxes of mitochondria from stromal cells to diseased cells. These mechanisms have been partially elucidated by Ahmad *et al.* by using both an *in vitro* model of co-culture of MSCs with epithelial cells and an *in vivo* mouse model of acute lung injury treated with MSCs administered via the trachea [147]. They demonstrated for the first time that mitochondrial transfer was a well-directed process regulated by the mitochondrial Rho-GTPase 1 Miro1 [147]. Consistent with this, previous studies have shown that Miro1 is a calcium-sensitive protein and that it acts as a surface receptor that drives the movement of mitochondria along microtubules by ensuring their attachment to adaptor trafficking kinesin proteins and motor proteins such as kinesin heavy chain and dynein in the cytoskeleton [148,149]. Interestingly, MSCs, which are considered potent donors of mitochondria, were found to express high levels of Miro1 compared with other cell types, such as lung epithelial cells or fibroblasts, both of which are considered poorer donors of mitochondria. Silencing Miro1 expression in stromal cells did not inhibit TNT formation, but it did seriously hamper mitochondria motility and rescue of injured somatic cells. In contrast, overexpression of Miro1 in MSCs increased their mitochondria donor capacity and, as a consequence, their rescue ability [148–150]. The discovery of the role of Miro1 in the transfer of mitochondria from stromal cells renders this protein a promising target for improving future MSC therapies.

Although one of the critical downstream players of mitochondrial transfer has been identified, signaling pathways initiating mitochondrial transfer in the recipient and donor cells remain to be elucidated. Further insights in this field may also contribute to maximizing mitochondrial transfer and mesenchymal-mediated cytoprotective functions.

In addition to mitochondria, other organelles, such as lysosomes or ribosomes, may also be transferred along TNT structures from MSCs to damaged cardiomyocytes. Indeed, uptake of exogenous lysosomes might increase the autophagic activity of stressed cardiomyocytes and thus increase their ability to eliminate their own damaged mitochondria in a similar fashion to that previously reported in mixed cultures between progenitors and endothelial cells [91]. Transfer of ribosomes together with mRNA could trigger protein synthesis in cardiac cells to confer resistance to future injury. A similar phenomenon has been recently described in a co-culture setting between Schwann cells and injured neurons [151,152]. Although it is clearly evident that transfer of organelles contributes to survival and/or rejuvenation of injured cells and that, because of the large size of these compounds, only TNTs might be able to mediate their traffic, we can speculate that other ways of cell-to-cell communication might be involved in the reprogramming process of dying cardiomyocytes. In particular, we can speculate that paracrine factors might be produced by MSCs that favor dedifferentiation/rejuvenation of cardiomyocytes. Among these factors, the anti-inflammatory cytokine of the IL-6 family, oncostatin M, has recently been reported to be involved in the rejuvenation both *in vitro* and *in vivo* of mammalian cardiomyocytes that highly expressed oncostatin M receptors [141]. However, whether this cytokine is produced by MSCs following cell-to-cell communication with injured cardiomyocytes remains to be determined. Additionally, genetic material of MSC origin, such as miRNAs, could contribute to the somatic reprogramming of cardiac cells. In such circumstances, genetic material may be enclosed in exosomes and microvesicles, which might be secreted in the extracellular space by donor cells or transported along TNT structures [94] or gap junctions [57]. Further studies are required to identify the different modalities and mediators of cell-to-cell communication between stromal cells and cardiac cells and their respective roles in the cardiomyocyte reprogramming process. Finally, in the attempt to extrapolate observations made in the heart, it would be interesting to determine whether MSCs might be able to rejuvenate other post-mitotic cells with poor self-renewal capability, such as neurons. New insights on the cytoprotective role of stromal cell mitochondria, the mechanisms of TNT formation, and the factors governing the reprogramming will be useful in the field of regenerative medicine for improving treatments against such illnesses as myocardial infarction and brain ischemia.

### 21.4.3 Tunneling nanotube cell-to-cell communication with distressed cardiomyocytes stimulates the paracrine repair function of mesenchymal stromal cells

Although the consequences of TNT-mediated cell-to-cell communication between MSCs and damaged cells is beginning to be better characterized in both *in vitro* and *in vivo* experimental settings, little is known as to whether this process affects the behavior of MSCs.

Initial *in vitro* studies indicated that xenogeneic TNT connections between MSCs and cardiomyocytes enabled MSCs to acquire expression of some cardiac-specific markers, and thus

to adopt a cardiomyogenic-like fate [86,87]. However, these early studies were conducted with healthy neonatal cardiomyocytes and were unsuitable for determining the impact of TNT bridging on MSC functions when adult damaged cardiomyocytes were used. This issue was recently addressed by Figeac *et al.* [89] through the use of an *in vitro* system of co-culture between human multipotent adipose-tissue-derived stromal cells in a murine MSC model involving dying adult murine cardiomyocytes. In the experimental setting developed by Figeac *et al.*, no differentiation of MSCs into cardiomyocytes was observed, confirming that occurrence of this phenomenon relies on the developmental (neonatal versus adult) and/or the physiological state (healthy versus damaged) of somatic cells. The authors observed that xenogeneic (heterologous) TNT connections altered the secretome of MSCs. Specifically, Luminex quantification of the release of 20 cytoprotective soluble factors by MSCs indicated that vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), stromal cell-derived factor-1 (SDF-1, also known as CXCL12), and monocyte chemoattractant protein (MCP)-3 were overexpressed in MSCs following co-culture with distressed cardiomyocytes through a mechanism dependent on TNT crosstalk. (Luminex's multiplex bead-based immunoassay testing platform simultaneously measures multiple analytes by exciting a sample with a laser and subsequently analyzing the wavelength of emitted light.) Additional *in vitro* experiments addressed the biological significance of these paracrine changes and clearly revealed that TNT-mediated cell-to-cell communications significantly increased the paracrine capacity of MSCs to promote angiogenesis and chemotaxis of bone-marrow-derived progenitor cells. Furthermore, Figeac *et al.* [89] used neutralizing antibodies to perform angiogenesis and chemotaxis assays and found that increased secretion of VEGF and HGF was responsible for the improved angiogenesis activity of co-cultivated MSCs, while the upregulated secretion of VEGF, HGF, SDF-1, and MCP-3 participated in recruitment of bone-marrow-derived progenitor cells. This study provided the first demonstration in a cardiac model that the secretion of MSCs is regulated by the local microenvironment (i.e., in this case, injured cardiomyocytes) and that TNT structures play a critical role by improving the angiogenesic and chemotactic capacities of MSCs. These two processes are of key importance in wound healing by providing oxygen and nutrient supply to the site of injury and by attracting bone marrow stem/progenitor cells with similar regenerative effects to the site of injury [50–53]. Thus, it is noteworthy that, in Figeac *et al.*'s study, TNT-mediated cell-to-cell communications with damaged cardiomyocytes did not apparently modify the paracrine action of MSCs exerted on the proliferation of human primary cardiac progenitor cells, the protection of cardiomyocytes against serum withdrawal-induced apoptosis, or the inhibition of fibroblast proliferation and collagen synthesis in the heart. Nevertheless, these findings do not preclude direct intercellular communication processes that include paracrine signaling or exchanges of cellular components of MSCs with cardiac fibroblasts or resident progenitor cells that could ameliorate their regenerative capacities related to inhibition of fibrosis and stimulation of progenitor proliferation. Indeed, we have already discussed direct cell-to-cell communications of MSCs with damaged cardiomyocytes that could improve their survival.

Whether TNT-mediated cell-to-cell communication improves the paracrine repair function of MSCs was confirmed by *in vivo* studies carried out in a mouse model of myocardial infarction using naive versus co-cultivated MSCs. Consistent with *in vitro* findings, MSCs previously co-cultured with dying cardiomyocytes were found to exhibit greater regenerative capacity than naive MSCs. Mice treated with co-cultivated cells displayed better cardiac contractile function associated with enhanced angiogenesis and homing of bone-marrow-derived progenitor cells to the myocardial injury site, compared with mice treated with naive MSCs. Strikingly, the increased therapeutic effectiveness of co-cultured MSCs was found not to be due to higher production of cardioprotective factors than naive ones at the time of intramyocardial delivery of the cells after 4 days of co-culture. These surprising results indicated that alteration of the MSC secretome mediated by xenogeneic TNTs was transient. Although it remains to be determined why this phenomenon is ephemeral, we can speculate that, at this later stage of co-culturing, co-cultured cardiomyocytes were mainly dead, rendering TNT bridging with MSCs impossible.

In contrast to the relative lack of secretome differences between naive and co-cultivated MSCs at later stages of co-culture, it is interesting to note that a second exposure of co-cultured MSCs to cardiomyocytes, after injection into the heart, increased *de novo* their release of TNT-dependent cytokines. The activation of the MSC secretome was shown to be more efficient after the second cardiomyocyte priming than after the first one and was dependent on the cardiomyocyte to MSC ratio. These observations suggest that activation of the MSC secretome following MSCs injection into the heart might be expected to be greater than its activation *in vitro,* since MSCs should encounter a broader range of cardiomyocytes *in vivo*.

Despite the lack of formal demonstration that TNT interactions with dying cardiomyocytes increase the regenerative properties of co-cultured MSCs *in vivo*, TNT-mediated communication between stromal cells and cardiac cells probably occurs *in vivo* since mitochondrial exchanges from stromal cells to myocardial cells were found in areas of murine myocardial infarcts that had been injected with MSCs.

Beyond the importance of TNT connections with cardiomyocytes in improving MSC paracrine function, we cannot exclude the possibility that the increased therapeutic efficacy of co-cultured MSCs *in vivo* could also result from cell-to-cell communications of injected MSCs with other cells resident in the heart, such as fibroblasts, endothelial cells, or cardiac progenitors. MSCs could communicate with these cells through TNT

structures or by other mechanisms, including gap junctions and paracrine factors. Such phenomena might maximize the healing of cardiac damage. Additionally, it is also possible that injected MSCs could directly interact with immune cells to minimize the deleterious side effects triggered by acute inflammation, which is a key component of infarcted myocardium. Whether TNT-mediated cell-to-cell communications could be involved in the modulation of inflammatory processes mediated by MSCs is unknown, but we can speculate that TNTs might activate the secretion of anti-inflammatory cytokines and decrease the secretion of pro-inflammatory cytokines by MSCs, following their interaction with damaged cardiomyocytes and other injured cells resident in the heart. Consistent with this hypothesis, TNT formation between stromal and cardiac cells has been shown to be sensitive to inflammatory stimuli, since treatment with TNF-α or IFN-γ increased the number of xenogeneic connections and the transcription of TNT-dependent cytokines such as VEGF, HGF, MCP-3, and SDF-1 by MSCs. Modulation of the inflammatory response to injury combined with potential direct interactions of MSCs with immune cells, such as macrophages, dendritic cells, or lymphocytes, might favor a phenotypic switch of these cells of the immune system from a pro-inflammatory phenotype to an anti-inflammatory phenotype.

Direct implication of TNT effects is difficult to assess *in vivo,* since these structures are transient and very fragile. Future studies could address this issue indirectly by comparing the efficacy of MSC administration as a function of increasing doses of cells administered, and/or by using different routes of MSC delivery. For example, MSCs injected intravenously may be unable to interact with cardiomyocytes because they may be retained in the lungs, thus minimizing the potential involvement of TNTs in the heart.

Finally, an important but so far unsolved question concerns the nature of the stress cues sent by cardiomyocytes along TNTs that trigger the paracrine activation of MSCs. We can speculate that miRNA, proteins, or organelles, such as lysosomes or mitochondria, likely contribute to different extents to TNT-mediated cell signaling. In addition, upstream signals initiating TNT formation during the co-culture between stromal cells and cardiac cells must be elucidated. In order to complete the work conducted by Figeac *et al.* [89], it is clearly evident that cell-to-cell communication pathways, other than TNT crosstalk, include soluble mediators, exosomes, large microvesicles, and apoptotic bodies secreted by cardiomyocytes and engulfed by MSCs. These factors also contribute to the modification of MSC paracrine activity. Diversity of the mechanisms by which MSCs and cardiomyocytes communicate during co-culture was illustrated by the decreased release of MCP-1 and MCP-3 in co-cultured MSCs, even when physical contact between the two cell types was impeded or when there was increased stromal cell release of IL-6 and growth-related protein (GRO)-1α. These observations strongly suggested that uptake by MSCs of large microparticles (>0.4 μm) [153] or apoptotic bodies of cardiac origin [62,154] might stimulate the release of IL-6 and GRO-1α, while soluble factors or exosomes/small microvesicles [155] might decrease the production of matrix metalloproteinase (MMP)-3 and MCP-1. However, despite their occurrence, these communication pathways do not appear to be responsible of the increased angiogenic and chemotactic properties of MSCs following after co-culture. We can speculate that release of IL-6 and GRO-1α by co-cultured MSCs indirectly enhances their pro-angiogenic effects by activating the secretion of VEGF and/or the myocardial homing of bone-marrow-derived endothelial progenitors [156,157].

Concomitant with the increased production of soluble factors, the enhanced effects of co-cultured MSCs may also be due to downregulation of harmful diffusible molecules that contribute to pathological left ventricular remodelling after AMI. These include MCP-1, previously reported to exacerbate myocardial inflammation [158], and MMP-3, incriminated in extracellular matrix degradation [159].

In conclusion, further studies are required to formally determine the extent of modification of the MSC secretome following intercellular communication with distressed cardiomyocytes and, in particular, the identification of additional soluble factors involved in healing processes. The principal findings of this section of the chapter are summarized in Figure 21.9.

## 21.5 Conclusions

Cell therapies based on the use of MSCs constitute a promising tool to ameliorate damaged cardiac muscle. Various clinical trials have revealed that delivery of MSCs was feasible and safe. Nevertheless, the clinical benefit of MSC treatment in some trials has been reported to be insufficient to provide long-term patient survival. Thus, intensive efforts are currently being devoted to optimizing the therapeutic efficacy of MSCs. Reaching this goal probably resides in a deeper understanding of the cell-to-cell communication processes between engrafted stromal cells and resident cardiac cells since MSCs activate their regenerative functions in contact with stressed cells in the microenvironment.

Among the diversity and complexity of intercellular crosstalk, TNT structures are particularly fascinating. Emerging data indicate that these structures are important for the rescue by stromal cells of dying somatic cells through the transfer of organelles (and probably other compounds) or for activating the paracrine pro-angiogenic and chemoattractant functions of MSCs. Further investigations are clearly needed to elucidate all facets of TNTs in controlling regenerative processes. With current insights it is likely that TNTs control inflammatory processes and maximize tissue damage by allowing stromal cells to interact with a broad ranges of cell types over long distances.

Future studies on this field may enable investigators to stimulate TNT formation and thereby further improve intercellular exchanges involved in the therapeutic effectiveness of MSCs.

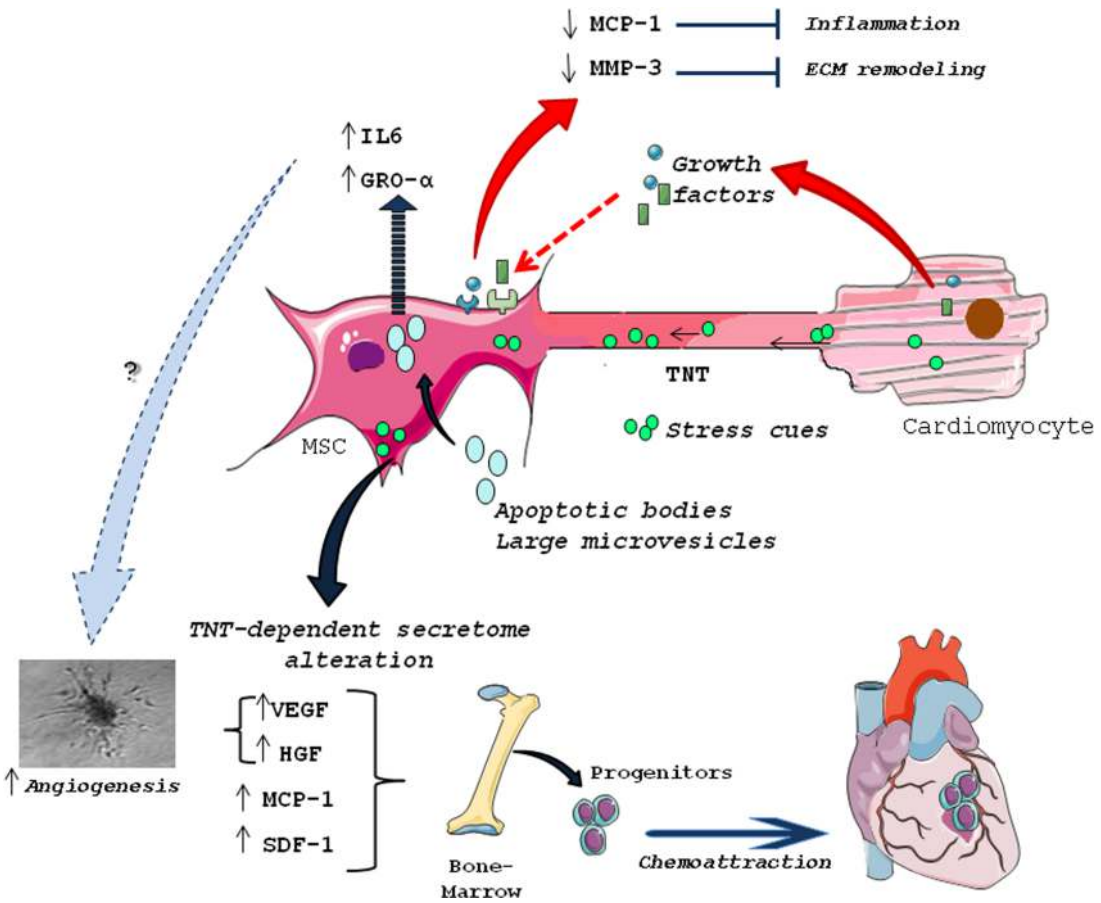



**Figure 21.9** TNT cell-to-cell communication with dying cardiomyocytes alters the secretome of MSCs. Schema summarizing TNT structures improving the paracrine, angiogenic, and chemoattractant functions of MSCs. Other pathways of cell-to-cell communications are also represented as affecting the MSC secretome. This figure was produced using Servier Medical Art.

## References


1 M. Tendera and W. Wojakowski. Clinical trials using autologous bone marrow and peripheral blood-derived progenitor cells in patients with acute myocardial infarction. *Folia Histochem Cytobiol* 2005;**43**(4):233–235.

2 J. S. Burchfield, M. Xie, and J. A. Hill. Pathological ventricular remodeling: mechanisms: part 1 of 2. *Circulation* 2013;**128**(4):388–400.

3 M. Xie, J. S. Burchfield, and J. A. Hill. Pathological ventricular remodeling: therapies: part 2 of 2. *Circulation* 2013;**128**(9):1021–1030.

4 J. P. Bassand, C. W. Hamm, D. Ardissino, *et al.* Guidelines for the diagnosis and treatment of non-ST-segment elevation acute coronary syndromes. *Eur Heart J* 2007;**28**(13):1598–1660.

5 F. van de Werf, J. Bax, A. Betriu, *et al.* Management of acute myocardial infarction in patients presenting with persistent ST-segment elevation: the Task Force on the Management of ST-Segment Elevation Acute Myocardial Infarction of the European Society of Cardiology. *Eur Heart J* 2008;**29**(23):2909–2945.

6 V. F. Segers and R. T. Lee. Stem-cell therapy for cardiac disease. *Nature* 2008;**451**(7181):937–942.

7 J. Tongers, D. W. Losordo, and U. Landmesser. Stem and progenitor cell-based therapy in ischaemic heart disease: promise, uncertainties, and challenges. *Eur Heart J* 2011;**32**(10):1197–1206.

8 S. Janssens. Stem cells in the treatment of heart disease. *Annu Rev Med* 2010;**61**:287–300.

9 P. Menasché, A. A. Hagège, J. T. Vilquin, *et al.* Autologous skeletal myoblast transplantation for severe postinfarction left ventricular dysfunction. *J Am Coll Cardiol* 2003;**41**(7):1078–1083.

10 P. C. Smits, R. J. van Geuns, D. Poldermans, *et al.* Catheter-based intramyocardial injection of autologous skeletal myoblasts as a primary treatment of ischemic heart failure: clinical experience with six-month follow-up. *J Am Coll Cardiol* 2003;**42**(12):2063–2069.

11 J. J. Gavira, G. Abizanda, M. Pérez-Ilzarbe, *et al.* Skeletal myoblasts for cardiac repair in animal models. *Eur Heart J Suppl* 2008;**10**(Suppl K):K11–K15.

12 A. C. Fijnvandraat, A.C van Ginneken, C. A. Schumacher, *et al.* Cardiomyocytes purified from differentiated embryonic stem cells exhibit characteristics of early chamber myocardium. *J Mol Cell Cardiol* 2003;**35**(12):1461–1472.

13 I. Kehat, D. Kenyagin-Karsenti, M. Snir, *et al.* Human embryonic stem cells can differentiate into myocytes with structural and

functional properties of cardiomyocytes. *J Clin Invest* 2001;**108** (3):407–414.

14 M. G. Klug, M. H. Soonpaa, G. Y. Koh, and L. J. Field. Genetically selected cardiomyocytes from differentiating embronic stem cells form stable intracardiac grafts. *J Clin Invest* 1996;**98**(1):216–224.

15 S. Etzion, R. Holbova, L. Miller, and J. Leor. Protocols for myocardial infarction repair using fetal cardiac myocytes. *Methods Mol Med* 2005;**112**:205–221.

16 K. Okita, T. Ichisaka, and S. Yamanaka. Generation of germline-competent induced pluripotent stem cells. *Nature* 2007;**448**(7151):313–317.

17 K. Takahashi, K. Tanabe, M. Ohnuki, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**(5):861–872.

18 K. Takahashi, and S. Yamanaka. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**(4):663–676.

19 M. Wernig, A. Meissner, R. Foreman, *et al. In vitro* reprogramming of fibroblasts into a pluripotent ES-cell-like state. *Nature* 2007;**448** (7151):318–324.

20 A. I. Caplan and S. P. Bruder. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* 2001;**7**(6):259–264.

21 K. Fukuda. Use of adult marrow mesenchymal stem cells for regeneration of cardiomyocytes. *Bone Marrow Transplant* 2003;**32**(Suppl 1):S25–S27.

22 D. Hakuno, K. Fukuda, S. Makino, *et al.* Bone marrow-derived regenerated cardiomyocytes (CMG cells) express functional adrenergic and muscarinic receptors. *Circulation* 2002;**105**(3): 380–386.

23 S. Makino, K. Fukuda, S. Miyoshi, *et al.* Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J Clin Invest* 1999;**103** (5):697–705.

24 S. Mohanty, S. Bose, K. G. Jain, *et al.* TGFβ1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells. *Int J Cardiol* 2013;**163**(1):93–99.

25 A. Williams, X. Sun, J. E. Fischer, and P. O. Hasselgren. The expression of genes in the ubiquitin-proteasome proteolytic pathway is increased in skeletal muscle from patients with cancer. *Surgery* 1999;**126**(4):744–749.

26 S. Gronthos, M. Mankani, J. Brahim, *et al.* Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;**97**(25):13625–13630.

27 C. De Bari, F. Dell'Accio, P. Tylzanowski, and F. P. Luyten. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* 2001;**44**(8):1928–1942.

28 J. Kajstura, M. Rota, S. R. Hall, *et al.* Evidence for human lung stem cells. *N Engl J Med* 2011;**364**(19):1795–1806.

29 A. Erices, P. Conget, and J. J. Minguell. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;**109** (1):235–242.

30 P. S. In 't Anker, S. A. Scherjon, C. Kleijburg-van der Keur, *et al.* Amniotic fluid as a novel source of mesenchymal stem cells for therapeutic transplantation. *Blood* 2003;**102**(4):1548–1549.

31 J. G. Toma, M. Akhavan, K. J. Fernandes, *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001;**3**(9):778–784.

32 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284** (5411):143–147.

33 P. A. Zuk, M. Zhu, H. Mizuno, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**(2):211–228.

34 J. Gimble and F. Guilak. Adipose-derived adult stem cells: isolation, characterization, and differentiation potential. *Cytotherapy* 2003;**5** (5):362–369.

35 A. M. Rodriguez, C. Elabd, F. Delteil, *et al.* Adipocyte differentiation of multipotent cells established from human adipose tissue. *Biochem Biophys Res Commun* 2004;**315**(2):255–263.

36 F. P. Barry and J. M. Murphy. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;**36**(4):568–584.

37 G. D'Ippolito, S. Diabira, G. A. Howard, *et al.* Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 2004;**117**(Pt 14):2971–2981.

38 A. M. Rodriguez, D. Pisani, C. A. Dechesne, *et al.* Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J Exp Med* 2005;**201**(9):1397–1405.

39 K. Le Blanc. Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 2003;**5**(6):485–489.

40 J. G. Shake, P. J. Gruber, W. A. Baumgartner, *et al.* Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* 2002;**73** (6):1919–1925.

41 L. Zeng, Q. Hu, X. Wang, *et al.* Bioenergetic and functional consequences of bone marrow-derived multipotent progenitor cell transplantation in hearts with postinfarction left ventricular remodeling. *Circulation* 2007;**115**(14):1866–1875.

42 J. M. Zimmet and J. M. Hare. Emerging role for bone marrow derived mesenchymal stem cells in myocardial regenerative therapy. *Basic Res Cardiol* 2005;**100**(6):471–481.

43 J. O. Beitnes, K. Lunde, J. E. Brinchmann, and S. Aakhus. Stem cells for cardiac repair in acute myocardial infarction. *Expert Rev Cardiovasc Ther* 2011;**9**(8):1015–1025.

44 D. Orlic, J. Kajstura, S. Chimenti, *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* 2001;**410**(6829):701–705.

45 C. Toma, M. F. Pittenger, K. S. Cahill, *et al.* Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002;**105**(1):93–98.

46 S. Tomita, D. A. Mickle, R. D. Weisel, *et al.* Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg* 2002;**123**(6):1132–1140.

47 M. Alvarez-Dolado, R. Pardal, J. M. Garcia-Verdugo, *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* 2003;**425**(6961):968–973.

48 J. M. Nygren, S. Jovinge, M. Breitbach, *et al.* Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nat Med* 2004;**10** (5):494–501.

49 J. M. Hill, A. J. Dick, V. K. Raman, *et al.* Serial cardiac magnetic resonance imaging of injected mesenchymal stem cells. *Circulation* 2003;**108**(8):1009–1014.

50 M. Gnecchi, H. He, O. D. Liang, *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;**11**(4):367–368.

51 M. Huang, P. Nguyen, F. Jia, *et al.* Double knockdown of prolyl hydroxylase and factor-inhibiting hypoxia-inducible factor with nonviral minicircle gene therapy enhances stem cell mobilization and angiogenesis after myocardial infarction. *Circulation* 2011;**124** (11 Suppl):S46–S54.

52 R. Uemura, M. Xu, N. Ahmad, and M. Ashraf. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. *Circ Res* 2006;**98**(11):1414–1421.

53 K. R. Vrijsen, S. A. Chamuleau, W. A. Noort, *et al.* Stem cell therapy for end-stage heart failure: indispensable role for the cell? *Curr Opin Organ Transplant* 2009;**14**(5):560–565.

54 F. van den Akker, S. C. de Jager, and J. P. Sluijter. Mesenchymal stem cell therapy for cardiac inflammation: immunomodulatory properties and the influence of toll-like receptors. *Mediators Inflamm* 2013;**2013**:181020.

55 D. I. Cho, M. R. Kim, H. Y. Jeong, *et al.* Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med* 2014;**46**:e70.

56 S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 2012;**10** (3):244–258.

57 T. Hosoda, H. Zheng, M. Cabral-da-Silva, *et al.* Human cardiac stem cell differentiation is regulated by a mircrine mechanism. *Circulation* 2011;**123**(12):1287–1296.

58 F. Li, Q. Huang, J. Chen, *et al.* Apoptotic cells activate the "phoenix rising" pathway to promote wound healing and tissue regeneration. *Sci Signal* 2010;**3**(110):ra13.

59 C. Popov, T. Radic, F. Haasters, *et al.* Integrins $\alpha_2\beta_1$ and $\alpha_{11}\beta_1$ regulate the survival of mesenchymal stem cells on collagen I. *Cell Death Dis* 2011;**2**:e186.

60 J. Du, X. Chen, X. Liang, *et al.* Integrin activation and internalization on soft ECM as a mechanism of induction of stem cell differentiation by ECM elasticity. *Proc Natl Acad Sci U S A* 2011;**108**(23):9466–9471.

61 A. J. Engler, S. Sen, H. L. Sweeney, and D. E. Discher. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126** (4):677–689.

62 M. Hristov, W. Erl, S. Linder, and P. C. Weber. Apoptotic bodies from endothelial cells enhance the number and initiate the differentiation of human endothelial progenitor cells *in vitro*. *Blood* 2004;**104**(9):2761–2766.

63 J. P. Sluijter, V. Verhage, J. C. Deddens, *et al.* Microvesicles and exosomes for intracardiac communication. *Cardiovasc Res* 2014;**102**(2):302–311.

64 K. Ekström, O. Omar, C. Granéli, *et al.* Monocyte exosomes stimulate the osteogenic gene expression of mesenchymal stem cells. *PLoS ONE* 2013;**8**(9):e75227.

65 R. Hass and A. Otte. Mesenchymal stem cells as all-round supporters in a normal and neoplastic microenvironment. *Cell Commun Signal* 2012;**10**(1):26.

66 J Gu, H. Qian, L. Shen, *et al.* Gastric cancer exosomes trigger differentiation of umbilical cord derived mesenchymal stem cells to carcinoma-associated fibroblasts through TGF-β/Smad pathway. *PLoS ONE* 2012;**7**(12):e52465.

67 K. Anton, D. Banerjee, and J. Glod. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. *PLoS ONE* 2012;**7**(4):e35036.

68 C. Corrado, S. Raimondo, L. Saieva, *et al.* Exosome-mediated crosstalk between chronic myelogenous leukemia cells and human bone marrow stromal cells triggers an interleukin 8-dependent survival of leukemia cells. *Cancer Lett* 2014;**348**(1–2):71–76.

69 H. C. Zhang, X. B. Liu, S. Huang, *et al.* Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev* 2012;**21**(18):3289–3297.

70 K. Sabin and N. Kikyo. Microvesicles as mediators of tissue regeneration. *Transl Res* 2014;**163**(4):286–295.

71 A. Rustom, R. Saffrich, I. Markovic, *et al.* Nanotubular highways for intercellular organelle transport. *Science* 2004;**303**(5660):1007–1010.

72 N. V. Bukoreshtliev, X. Wang, E. Hodneland, *et al.* Selective block of tunneling nanotube (TNT) formation inhibits intercellular organelle transfer between PC12 cells. *FEBS Lett* 2009;**583**(9):1481–1488.

73 K. Gousset, E. Schiff, C. Langevin, *et al.* Prions hijack tunnelling nanotubes for intercellular spread. *Nat Cell Biol* 2009;**11**(3):328–336.

74 Y. Wang, J. Cui, X. Sun, and Y. Zhang. Tunneling-nanotube development in astrocytes depends on p53 activation. *Cell Death Differ* 2011;**18**(4):732–742.

75 E. A. Eugenin, P. J. Gaskill, and J. W. Berman. Tunneling nanotubes (TNT) are induced by HIV-infection of macrophages: a potential mechanism for intercellular HIV trafficking. *Cell Immunol* 2009;**254**(2):142–148.

76 K. Hase, S. Kimura, H. Takatsu, *et al.* M-Sec promotes membrane nanotube formation by interacting with Ral and the exocyst complex. *Nat Cell Biol* 2009;**11**(12):1427–1432.

77 B. Onfelt, S. Nedvetzki, R. K. Benninger, *et al.* Structurally distinct membrane nanotubes between human macrophages support long-distance vesicular traffic or surfing of bacteria. *J Immunol* 2006;**177** (12):8476–8483.

78 S. C. Watkins and R. D. Salter. Functional connectivity between immune cells mediated by tunneling nanotubules. *Immunity* 2005;**23**(3):309–318.

79 S. Sowinski, C. Jolly, O. Berninghausen, *et al.* Membrane nanotubes physically connect T cells over long distances presenting a novel route for HIV-1 transmission. *Nat Cell Biol* 2008;**10**(2):211–219.

80 X. Wang, M. L. Veruki, N. V. Bukoreshtliev, *et al.* Animal cells connected by nanotubes can be electrically coupled through interposed gap-junction channels. *Proc Natl Acad Sci U S A* 2010;**107** (40):17194–17199.

81 P. Tavi, T. Korhonen, S. L. Hänninen, *et al.* Myogenic skeletal muscle satellite cells communicate by tunnelling nanotubes. *J Cell Physiol* 2010;**223**(2):376–383.

82 D. Wittig, X. Wang, C. Walter, *et al.* Multi-level communication of human retinal pigment epithelial cells via tunneling nanotubes. *PLoS ONE* 2012;**7**(3):e33195.

83 A. Takahashi, A. Kukita, Y. J. Li, *et al.* Tunneling nanotube formation is essential for the regulation of osteoclastogenesis. *J Cell Biochem* 2013;**114**(6):1238–1247.

84 E. Lou, S. Fujisawa, A. Morozov, *et al.* Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. *PLoS ONE* 2012;**7**(3): e33093.

85 J. Pasquier, L. Galas, C. Boulangé-Lecomte, *et al.* Different modalities of intercellular membrane exchanges mediate cell-to-cell p-glycoprotein transfers in MCF-7 breast cancer cells. *J Biol Chem* 2012;**287**(10):7374–7387.

86 M. Koyanagi, R. P. Brandes, J. Haendeler, *et al.* Cell-to-cell connection of endothelial progenitor cells with cardiac myocytes by nanotubes: a novel mechanism for cell fate changes? *Circ Res* 2005;**96**(10):1039–1041.

87 E. Y. Plotnikov, T. G. Khryapenkova, A. K. Vasileva, *et al.* Cell-to-cell cross-talk between mesenchymal stem cells and cardiomyocytes in co-culture. *J Cell Mol Med* 2008;**12** (5A):1622–1631.

88 A. Acquistapace, T. Bru, P. F. Lesault, *et al.* Human mesenchymal stem cells reprogram adult cardiomyocytes toward a progenitor-like state through partial cell fusion and mitochondria transfer. *Stem Cells* 2011;**29**(5):812–824.

89 F. Figeac, P. F. Lesault, O. Le Coz, *et al.* Nanotubular crosstalk with distressed cardiomyocytes stimulates the paracrine repair function of mesenchymal stem cells. *Stem Cells* 2014;**32** (1):216–230.

90 K. Liu, K. Ji, L. Guo, *et al.* Mesenchymal stem cells rescue injured endothelial cells in an *in vitro* ischemia–reperfusion model via tunneling nanotube like structure-mediated mitochondrial transfer. *Microvasc Res* 2014;**92**:10–18.

91 K. Yasuda, A. Khandare, L. Burianovskyy, *et al.* Tunneling nanotubes mediate rescue of prematurely senescent endothelial cells by endothelial progenitors: exchange of lysosomal pool. *Aging* 2011;**3** (6):597–608.

92 E. Y. Plotnikov, T. G. Khryapenkova, S. I. Galkina, *et al.* Cytoplasm and organelle transfer between mesenchymal multipotent stromal cells and renal tubular cells in co-culture. *Exp Cell Res* 2010;**316** (15):2447–2455.

93 K. C. Vallabhaneni, H. Haller, and I. Dumler. Vascular smooth muscle cells initiate proliferation of mesenchymal stem cells by mitochondrial transfer via tunneling nanotubes. *Stem Cells Dev* 2012;**21**(17):3104–3113.

94 V. Thayanithy, E. L. Dickson, C. Steer, *et al.* Tumor–stromal cross talk: direct cell-to-cell transfer of oncogenic microRNAs via tunneling nanotubes. *Transl Res* 2014;**164**(5):359–365.

95 J. Pasquier, B. S. Guerrouahen, H. Al Thawadi, *et al.* Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med* 2013;**11**:94.

96 C. Pyrgaki, P. Trainor, A. K. Hadjantonakis, and L. Niswander. Dynamic imaging of mammalian neural tube closure. *Dev Biol* 2010;**344**(2):941–947.

97 E. Salas-Vidal and H. Lomelí. Imaging filopodia dynamics in the mouse blastocyst. *Dev Biol* 2004;**265**(1):75–89.

98 M. C. McKinney, D. A. Stark, J. Teddy, and P. M. Kulesa. Neural crest cell communication involves an exchange of cytoplasmic material through cellular bridges revealed by photoconversion of KikGR. *Dev Dyn* 2011;**240**(6):1391–1401.

99 T. H. Millard and P. Martin. Dynamic analysis of filopodial interactions during the zippering phase of *Drosophila* dorsal closure. *Development* 2008;**135**(4):621–626.

100 L. Caneparo, P. Pantazis, W. Dempsey, and S. E. Fraser. Intercellular bridges in vertebrate gastrulation. *PLoS ONE* 2011;**6**(5): e20230.

101 H. R. Chinnery, E. Pearlman, and P. G. McMenamin. Cutting edge: membrane nanotubes *in vivo*: a feature of MHC class $II^+$ cells in the mouse cornea. *J Immunol* 2008;**180**(9):5779–5783.

102 Y. Seyed-Razavi, M. J. Hickey, L. Kuffová, *et al.* Membrane nanotubes in myeloid cells in the adult mouse cornea represent a novel mode of immune cell interaction. *Immunol Cell Biol* 2013;**91** (1):89–95.

103 X. Li, Y. Zhang, S. C. Yeung, *et al.* Mitochondrial transfer of induced pluripotent stem cell-derived mesenchymal stem cells to airway epithelial cells attenuates cigarette smoke-induced damage. *Am J Respir Cell Mol Biol* 2014;**51**(3):455–465.

104 W. W. Minuth and L. Denk. Cell projections and extracellular matrix cross the interstitial interface within the renal stem/progenitor cell niche: accidental, structural or functional cues? *Nephron Exp Nephrol* 2012;**122**(3–4):131–140.

105 X. Wang, N. V. Bukoreshtliev, and H.-H. Gerdes. Developing neurons form transient nanotubes facilitating electrical coupling and calcium signaling with distant astrocytes. *PLoS ONE* 2012;**7** (10):e47429.

106 M. W. Austefjord, H.-H. Gerdes, and X. Wang. Tunneling nanotubes: diversity in morphology and structure. *Commun Integr Biol* 2014;**7**(1):e27934.

107 Z. G. Wang, S. L. Liu, Z.Q. Tian, *et al.* Myosin-driven intercellular transportation of wheat germ agglutinin mediated by membrane nanotubes between human lung cancer cells. *ACS Nano* 2012;**6** (11):10033–10041.

108 K. He, W. Luo, Y. Zhang, *et al.* Intercellular transportation of quantum dots mediated by membrane nanotubes. *ACS Nano* 2010;**4**(6):3015–3022.

109 A. Chauveau, A. Aucher, P. Eissmann, *et al.* Membrane nanotubes facilitate long-distance interactions between natural killer cells and target cells. *Proc Natl Acad Sci U S A* 2010;**107**(12):5545–5550.

110 D. M. Davis and S. Sowinski. Membrane nanotubes: dynamic long-distance connections between animal cells. *Nat Rev Mol Cell Biol* 2008;**9**(6):431–436.

111 A. J. Ridley. Life at the leading edge. *Cell* 2011;**145**(7):1012–1022.

112 B. P. Jena. Role of SNAREs in membrane fusion. *Adv Exp Med Biol* 2011;**713**:13–32.

113 L. V. Chernomordik and M. M. Kozlov. Protein–lipid interplay in fusion and fission of biological membranes. *Annu Rev Biochem* 2003;**72**:175–207.

114 C. Schiller, K. N. Diakopoulos, I. Rohwedder, *et al.* LST1 promotes the assembly of a molecular machinery responsible for tunneling nanotube formation. *J Cell Sci* 2013;**126**(Pt 3):767–777.

115 P. D. Arkwright, F. Luchetti, J. Tour, *et al.* Fas stimulation of T lymphocytes promotes rapid intercellular exchange of death signals via membrane nanotubes. *Cell Res* 2010;**20**(1):72–88.

116 K. He, X. Shi, X. Zhang, *et al.* Long-distance intercellular connectivity between cardiomyocytes and cardiofibroblasts mediated by membrane nanotubes. *Cardiovasc Res* 2011;**92**(1):39–47.

117 S. Domhan, L. Ma, A. Tai, *et al.* Intercellular communication by exchange of cytoplasmic material via tunneling nano-tube like structures in primary human renal epithelial cells. *PLoS ONE* 2011;**6**(6):e21283.

118 S. Gurke, J. F. Barroso, and H.-H. Gerdes. The art of cellular communication: tunneling nanotubes bridge the divide. *Histochem Cell Biol* 2008;**129**(5):539–550.

119 I. F. Smith, J. Shuai, and I. Parker. Active generation and propagation of $Ca^{2+}$ signals within tunneling membrane nanotubes. *Biophys J* 2011;**100**(8):L37–L39.

120 J. M. Teddy and P. M. Kulesa. *In vivo* evidence for short- and long-range cell communication in cranial neural crest cells. *Development* 2004;**131**(24):6141–6151.

121 S. Chifflet, J. A. Hernández, and S. Grasso. A possible role for membrane depolarization in epithelial wound healing. *Am J Physiol Cell Physiol* 2005;**288**(6):C1420–C1430.

122 W. Wood, A. Jacinto, R. Grose, *et al.* Wound healing recapitulates morphogenesis in *Drosophila* embryos. *Nat Cell Biol* 2002;**4**(11):907–912.

123 C. Zhang, W. Xiong, H. Zheng, *et al.* Calcium- and dynamin-independent endocytosis in dorsal root ganglion neurons. *Neuron* 2004;**42**(2):225–236.

124 M. Levin. Large-scale biophysics: ion flows and regeneration. *Trends Cell Biol* 2007;**17**(6):261–270.

125 V. K. Rao and S. E. Straus. Causes and consequences of the autoimmune lymphoproliferative syndrome. *Hematology* 2006;**11**(1):15–23.

126 D. A. Martin, L. Zheng, R. M. Siegel, *et al.* Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia. *Proc Natl Acad Sci U S A* 1999;**96**(8):4552–4557.

127 F. Rieux-Laucat, S. Blachère, S. Danielan, *et al.* Lymphoproliferative syndrome with autoimmunity: a possible genetic basis for dominant expression of the clinical manifestations. *Blood* 1999;**94**(8):2575–2582.

128 Y. R. Chen. Mitochondrial dysfunction. In: F. A. Villamena (ed.), *Molecular Basis of Oxidative Stress: Chemistry, Mechanisms, and Disease Pathogenesis*. John Wiley & Sons, Inc.: Hoboken, NJ, 2013.

129 S. Naphade, J. Sharma, H. P. Gaide Chevronnay, *et al.* Lysosomal cross-correction by hematopoietic stem cell-derived macrophages via tunneling nanotubes. *Stem Cells* 2015;**33**(1):301–309.

130 C. Schiller, J. E. Huber, K. N. Diakopoulos, and E. H. Weiss. Tunneling nanotubes enable intercellular transfer of MHC class I molecules. *Hum Immunol* 2013;**74**(4):412–416.

131 I. Antanavičiūtė, K. Rysevaitė, V. Liutkevičius, *et al.* Long-distance communication between laryngeal carcinoma cells. *PLoS ONE* 2014;**9**(6):e99196.

132 C. Langevin, K. Gousset, M. Costanzo, *et al.* Characterization of the role of dendritic cells in prion transfer to primary neurons. *Biochem J* 2010;**431**(2):189–198.

133 K. Gousset and C. Zurzolo. Tunnelling nanotubes: a highway for prion spreading? *Prion* 2009;**3**(2):94–98.

134 A. Dedja, T. Zaglia, L. Dall'Olmo, *et al.* Hybrid cardiomyocytes derived by cell fusion in heterotopic cardiac xenografts. *FASEB J* 2006;**20**(14):2534–2536.

135 H. Oh, S. B. Bradfute, T. D. Gallardo, *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc Natl Acad Sci U S A* 2003;**100**(21):12313–12318.

136 T. R. Payne, H. Oshima, T. Sakai, *et al.* Regeneration of dystrophin-expressing myocytes in the mdx heart by skeletal muscle stem cells. *Gene Ther* 2005;**12**(16):1264–1274.

137 J. L. Spees, M. J. Whitney, D. E. Sullivan, *et al.* Bone marrow progenitor cells contribute to repair and remodeling of the lung and heart in a rat model of progressive pulmonary hypertension. *FASEB J* 2008;**22**(4):1226–1236.

138 R. B. Driesen, F. K. Verheyen, P. Dijkstra, *et al.* Structural remodelling of cardiomyocytes in the border zone of infarcted rabbit heart. *Mol Cell Biochem* 2007;**302**(1–2):225–232.

139 M. Szibor, J. Pöling, H Warnecke, *et al.* Remodeling and dedifferentiation of adult cardiomyocytes during disease and regeneration. *Cell Mol Life Sci* 2014;**71**(10):1907–1916.

140 J. Ausma, F. Thoné, G. D. Dispersyn, *et al.* Dedifferentiated cardiomyocytes from chronic hibernating myocardium are ischemia-tolerant. *Mol Cell Biochem* 1998;**186**(1–2):159–168.

141 T. Kubin, J. Pöling, S. Kostin, *et al.* Oncostatin M is a major mediator of cardiomyocyte dedifferentiation and remodeling. *Cell Stem Cell* 2011;**9**(5):420–432.

142 C. Jopling, E. Sleep, M. Raya, *et al.* Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* 2010;**464**(7288):606–609.

143 F. Laube, M. Heister, C. Scholz, *et al.* Re-programming of newt cardiomyocytes is induced by tissue regeneration. *J Cell Sci* 2006;**119**(Pt 22):4719–4729.

144 G. Grafi. How cells dedifferentiate: a lesson from plants. *Dev Biol* 2004;**268**(1):1–6.

145 A. Masuzawa, K. M. Black, C. A. Pacak, *et al.* Transplantation of autologously derived mitochondria protects the heart from ischemia–reperfusion injury. *Am J Physiol Heart Circ Physiol* 2013;**304**(7):H966–H982.

146 Z. Liu and R. A. Butow. Mitochondrial retrograde signaling. *Annu Rev Genet* 2006;**40**:159–185.

147 T. Ahmad, S. Mukherjee, B. Pattnaik, *et al.* Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. *EMBO J* 2014;**33**(9):994–1010.

148 S. Fransson, A. Ruusala, and P. Aspenström., The atypical Rho GTPases Miro-1 and Miro-2 have essential roles in mitochondrial trafficking. *Biochem Biophys Res Commun* 2006;**344**(2):500–510.

149 M. Saotome, D. Safiulina, G. Szabadkai, *et al.* Bidirectional $Ca^{2+}$-dependent control of mitochondrial dynamics by the Miro GTPase. *Proc Natl Acad Sci U S A* 2008;**105**(52):20728–20733.

150 G. Las and O. S. Shirihai. Miro1: new wheels for transferring mitochondria. *EMBO J* 2014;**33**(9):939–941.

151 F. A. Court, W. T. Hendriks, H. D. MacGillavry, *et al.* Schwann cell to axon transfer of ribosomes: toward a novel understanding of the role of glia in the nervous system. *J Neurosci* 2008;**28**(43):11024–11029.

152 F. A. Court, R. Midha, B. A. Cisterna, *et al.* Morphological evidence for a transport of ribosomes from Schwann cells to regenerating axons. *Glia* 2011;**59**(10):1529–1539.

153 R. C. Lai, F. Arslan, S. S. Tan, *et al.* Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J Mol Cell Cardiol* 2010;**48**(6):1215–1224.

154 S. Burghoff, Z. Ding, S. Gödecke, *et al.* Horizontal gene transfer from human endothelial cells to rat cardiomyocytes after intracoronary transplantation. *Cardiovasc Res* 2008;**77**(3):534–543.

155 G. Camussi, M. C. Deregibus, S. Bruno, *et al.* Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int* 2010;**78**(9):838–848.

156 A. A. Kocher, M. D. Schuster, N. Bonaros, *et al.* Myocardial homing and neovascularization by human bone marrow angioblasts is regulated by IL-8/Gro CXC chemokines. *J Mol Cell Cardiol* 2006;**40**(4):455–464.

157 T. Kinnaird, E. Stabile, M. S. Burnett, *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circ Res* 2004;**94**(5):678–685.

158 J. Niu and P. E. Kolattukudy. Role of MCP-1 in cardiovascular disease: molecular mechanisms and clinical implications. *Clin Sci (Lond)* 2009;**117**(3):95–109.

159 D. Kelly, S. Khan, G. Cockerill, *et al.* Circulating stromelysin-1 (MMP-3): a novel predictor of LV dysfunction, remodeling and all-cause mortality after acute myocardial infarction. *Eur J Heart Fail* 2008;**10**(2):133–139.

CHAPTER 22

# The preferential homing of mesenchymal stromal cells to sites of inflammation

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## 22.1 Introduction

In recent years there has been a dramatic increase in the potential use of mesenchymal stromal cell (MSC)-based therapy for the treatment of degenerative, inflammatory, autoimmune, and ischemic diseases. MSCs are present in numerous tissues throughout the body and can be isolated and expanded *in vitro* with ease. They are capable of multipotent differentiation and immune modulation [1]. Initial excitement in the MSC field centered on the potential of these cells to regenerate injured tissue; for example, to grow new cartilage or repair injured cerebral tissue. However, as research progressed it has become clear that it is through paracrine signaling rather than tissue regeneration that these cells exert their influence [2,3]. This has not dulled enthusiasm for the study of MSCs, as they have numerous advantageous characteristics. MSCs are easily and frequently isolated from bone marrow and adipose tissue, large banks of allogeneic MSCs can be stored, and autologous MSCs can be isolated in a relatively time-efficient manner. MSCs interact with a wide array of other cell populations to contribute to repair and have the ability to migrate under appropriate stimuli to sites of inflammation.

An important initial consideration is whether or not native MSCs migrate to sites of injury or if they are generated and act locally. Several studies have failed to detect MSCs in the systemic circulation [4,5], supporting the local tissue location hypothesis. In solid organ transplants, MSCs present in the donor tissue are consistently of donor origin with no evidence that host MSCs are recruited. One would expect that a transplanted organ would provide enough inflammatory stimuli for host MSC migration, although of course this may be confounded by the use of immunosuppressive medication posttransplant [6]. MSCs have, however, been reported in the peripheral circulation of a minority of hip fracture patients and in just under 50% of young traumatic fracture patients, suggesting that bone fracture might be sufficient stimulus to initiate *in vivo* migration of endogenous MSCs. Alternatively, it may simply reflect mechanical disruption of bone tissue, as occurs in fat embolization following trauma [7,8]. Circulating MSCs have also been reported in rats exposed to chronic hypoxia and in liver-injured mice [9,10].

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What remains unclear is why systemic recruitment of MSCs is necessary when they can be found locally in many tissues and if the immunophenotype of endogenous MSCs is the same as culture-expanded plastic adherent cells. Furthermore, if MSCs are recruited systemically they may not necessarily move through the systemic circulation as there has been a report suggesting that adipose-tissue-derived MSCs (AMSCs) migrate via lymphatics [11]. There are benefits to developing therapeutic agents to enhance homing of endogenous MSCs since neither extensive *ex vivo* manipulation nor a vehicle for delivery is needed, and this may be more acceptable in terms of safety.

Endogenous reservoirs may be depleted by age, physical illness, degenerative disease, physiological aberrations, or medications, which are the very groups of patients who would potentially benefit from the actions of MSCs. Thus, the primary focus will remain on the role of exogenously delivered MSCs in disease models. There have been studies of MSC administration in a variety of disease models, including arthritis [12], osteogenesis imperfecta [13], pancreatic islet repair [14], cardiac ischemia [15], inflammatory bowel disease [16], and ischemic stroke [17].

To maximize the potential benefit of MSCs, the mechanism of their ability to migrate to sites of injury needs to be more fully understood. There have been significant developments in identifying the chemokine receptors on MSCs and the role that they play in homing; in particular, the interaction between C–X–C motif chemokine receptor (CXCR)4 and stromal derived factor-1 (SDF-1) has been studied extensively, but other chemokines, such as monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein-1α (MIP-1α), as well as growth factors, have been studied and will be reviewed in this chapter. The contribution of the inflammatory environment itself needs to be considered, with particular focus on the proliferation of cytokines, influx of inflammatory cells, and activation of the complement system.

Initial *in vitro* work has led to a large range of *in vivo* homing studies with mixed results. Accurate comparisons between these studies are fraught with difficulty since the phenotype and immunophenotype of MSCs is known to vary with culture conditions, passage number, and source tissue. Indeed, the criteria for identifying MSCs as proposed by the Mesenchymal and Tissue Stromal Cell Committee of the International Society for Cellular Therapy was only published in 2006, and many studies predate this. These criteria are (1) plastic adherence of isolated cells in tissue culture; (2) >95% of the cultured cells positively expressing the cell surface markers CD105, CD73, and CD90, while being negative for CD34, CD45, CD14, CD11b, CD79a, CD19, and human leukocyte antigen-DR; and (3) *in vitro* differentiation into three mesodermal cell types, namely osteoblasts, adipocytes and chondroblasts [18]. In addition to inherent differences between MSC phenotypes, consideration needs to be given to the number of cells administered, the timing of this administration, the route used, and the detection method employed. This latter element poses significant challenges, and current procedures are getting away from *ex vivo* histological analysis and moving toward *in vivo* imaging, as will be discussed further.

Despite these difficulties great strides have been made in understanding cell migration and applying this knowledge to augment migration and improve disease outcomes through alterations in culture conditions, cell engineering strategies, and host modifications.

This chapter will review the major advances in the field of MSC migration *in vitro* and *in vivo*, focusing on the potential of exogenously delivered MSCs in the treatment of inflammatory and ischemic disease models.

## 22.2 Molecular mechanisms of migration

### 22.2.1 Chemokines

Chemokines have an important role to play in the migration of both endogenous and exogenous MSCs to sites of injury. Chemokines are small polypeptides and play a central role in the trafficking and localization of hematopoietic cells, as well as cell differentiation, survival, and activation [19–21]. It has been demonstrated that ligand binding to chemokine receptors can induce phosphorylation of mitogen activated protein kinases (MAPKs) such as extracellular signal-related kinase (ERK-1 and ERK-2) and activate focal adhesion kinase (FAK) pathways [22]. It is well known that chemokines act through MAPK pathways and that FAK activation has a central role in cell migration, adhesion, and survival [23,24]. Furthermore, C–X–C motif chemokine ligand (CXCL)12, the ligand for CXCR4, and chemokine (C–C motif) ligand (CCL)5, the ligand for chemokine receptor (CCR)1, activate STAT5 and STAT1 (signal transducer and activator of transcription) respectively. Stat pathways have important roles in cell differentiation and survival [22,25].

The role of chemokines in the migration of MSCs is important as it may provide insight into MSC function and provide targets for therapies designed to improve MSC migration to sites of injury. In one report, human bone-marrow-derived MSCs (BMSCs) were shown to express CCR1, CCR7, CCR9, CXCR4, CXCR5, and CXCR6 with no expression of CCR2–6, CCR8, and CXCR1–3 demonstrated [22,26]. However, CCR2, the receptor for monocyte chemoattractant protein 1 (MCP1 or CCL2) and CCR8, the receptor for interleukin (IL)-8, have been identified by other groups on murine MSCs, human periosteal progenitor cells, and human MSCs [27–29]. Rat MSCs have also been shown to express CXCXR4, CX3CR1, CCR2, and CCR5 [27,30].

Whether or not these chemokine receptors are functionally active on MSCs and are targeted by ligands produced by inflammation has been studied by various groups both *in vitro* and *in vivo*. In 2006, Honczarenko *et al.* reported that all identified chemokines, tested with the appropriate receptor, induced migration of bone marrow MSCs (BMSCs) *in vitro* with a bell-shaped dose-response relationship [22]. CCR2 and CCR8 may be activated to stimulate MSC migration both *in vitro* and to

breast cancer tissue *in vivo* [27–29]. IL-8 is known to play a role in the aggregation of neutrophils in inflamed tissue, and a chemotactic response to an IL-8 gradient has been demonstrated *in vitro*, suggesting it plays a role in MSC migration to inflamed tissue. However, robust *in vivo* data to corroborate this are lacking [31,32]. CCL2, also known as MCP-1, is another key chemokine that regulates migration and infiltration of monocytes and is upregulated in the inflammatory environment [33,34]. However, there are conflicting reports on the role of this chemokine in MSC migration studies, with one such study failing to demonstrate an *in vitro* MSC migratory response after MCP-1 stimulation [32]. MCP-3, or CCL7, has also emerged as a potentially important MSC homing factor, with improvement in cardiac function noted after serial infusions of MSCs in MCP-3-expressing hearts and associated overrecruitment of CCL7-recruited MSCs at the site of myocardial infarction [35].

MIP-1α, also known as CCL3, is a ligand for CCR1 and CCR5 and is known to regulate migration of proinflammatory cells to sites of inflammation. It can be upregulated by inflammatory stimuli from macrophages, dendritic cells, and lymphocytes, as is seen in the synovial fluid of patients with rheumatoid arthritis [36,37]. *In vitro* studies have demonstrated an increase in murine and human MSC migration in response to MIP-1α [38–40]. There is currently no information looking at the effect of MIP-1α or CCR1 inhibition or *in vivo* evidence to support MIP-1α as a chemoattractant to systemically delivered MSCs.

The role of SDF-1, also known at CXCL12, has been extensively studied in the recruitment of MSCs expressing the corresponding receptor CXCR4. CXCR4 is a G coupled transmembrane glycoprotein that is expressed on endothelial cells and which has been shown to mediate transcytosis of circulating SDF-1 into bone marrow with a resultant increase in the migration of human $CD34^+$ progenitor cells [41–43]. (Transcytosis is the process by which various macromolecules are transported across the interior of a cell. Macromolecules are captured in vesicles on one side of the cell, drawn across the cell, and ejected on the other side.)

Bone marrow endothelial cells, immature osteoblasts, and other bone marrow stromal cells constitutively express and secrete SDF-1 [44–46]. SDF-1 is found in particular in myelosupportive stromal cells near the endosteal surfaces of bone marrow [46–48]. In addition to endothelial and hematopoietic cell migration, SDF-1 regulates the hematopoietic cell cycle and also influences the survival and migration of neuronal, muscle, and liver cells [45,49–51].

In terms of injury models, SDF-1 expression is increased and peaks within 1 day at sites of myocardial infarction, is augmented in the kidney after ischemia–reperfusion-induced acute renal failure, and has been demonstrated at the site of hypoglossal nerve injury and in rat stroke models [30,52–54]. Further *in vitro* studies have attempted to confirm the role of CXCR4 activation in specific disease models. Healing live bone grafts *in vitro* showed high expression of SDF-1 in the periosteum with new bone formation inhibited by anti-SDF1 antibodies [55]. In an acute kidney injury (AKI) model, ischemia and reperfusion of renal tubular cells was used to mimic AKI *in vitro*: migration of BMSCs toward these injured cells was CXCR4 dependent, was increased by overexpression of CXCR4, and inhibited by a CXCR4-specific antagonist. Furthermore, phosphorylated AKT and MAPK was found to be increased in this AKI model and inhibition of these pathways partially blocked migration, suggesting that the SDF1-1/CXCR4 pathway acts through the P13K/AKT and MAPK pathways [56]. Therefore, it is hypothesized that the interaction between locally produced SDF-1 and CXCR4 expression on MSCs may facilitate migration of MSCs to the site of injury.

The SDF-1/CXCR4 interaction has been a particular focus of *in vitro* MSC migration studies to date. In one such study, BMSCs were harvested from healthy individuals, CXCR4 was identified with antibody staining, and CXCR4 mRNA confirmed by reverse transcriptase polymerase chain reaction (PCR). Only small percentages (1–3.9%) of cells were shown to express CXCR4 at the cell surface membrane. However, intracellular expression was confirmed in over 80% of cells. Cells were stimulated in transwell chambers with SDF-1, resulting in a dose-dependent increase in migration that was abrogated with the addition of anti-CXCR4 antibody [57]. Induced migration by activation of this pathway *in vitro* has also been demonstrated in human umbilical-cord-derived MSCs (UMSCs), which could be inhibited by antagonists of CXCR4 and inhibitors of P13K, MAPK, and p38 MAPK [58]. An abundance of SDF-1 transcripts has been demonstrated in human BMSCs with a preferential expression in immature stromal cell populations *in vitro* [59]. The expression of SDF-1 by this population of cells was shown to decrease as cells underwent osteogenesis. The high SDF-1 expression in immature stromal cells was shown to be protective against IL-4-induced apoptosis. Again, MSCs were shown to express low levels of functionally active cell surface CXCR4. It is likely that *in vivo* the SDF-1/CXCR4 interaction may function to localize immature stromal cells in a perivascular bone marrow niche and promote their survival until recruited elsewhere [59].

In addition to *in vitro* migration studies, stromal cells challenged with SDF-1 have been shown to undergo changes in F-actin polymerization [22]. By utilizing confocal microscopy, SDF-1 stimulation was shown to lead to an increase in intensity, orientation, and organization of intracellular filamentous networks. This is an early prolonged response that is thought to influence stromal cell migration [22].

Investigation into the role of the SDF-1/CXCR4 pathway has not been limited to *in vitro* migration but has been studied *in vivo* in several models, including neural tissue damage, cardiac ischemia, AKI, and bone repair [30,52,55,56,60,61]. Rat MSCs delivered intravenously were shown to migrate preferentially to areas in the cerebral cortex where recombinant human SDF-1 had been administered, while MSCs transplanted into the lateral ventricle of rats migrated to a unilateral avulsed (forcibly detached) hypoglossal nerve. This latter migratory effect was

associated temporally with increased levels of SDF-1 at the site of injury [30]. In the spinal cord, SDF-1 is expressed in the dorsal corticospinal tract and meninges, and both SDF-1 and CXCR4 are upregulated after traumatic spinal cord injury. Additionally, there are data suggesting that this pathway is implicated in the migration of MSCs to spinal cord injury after intrathecal or intravenous delivery [62–65]. In models of cardiac ischemia SDF-1 is secreted by injured cardiac cells, which may act as a chemoattractant for MSCs expressing CXCR4 [61]. This expression peaked within 24 h of injury, during which time intravenously delivered MSCs were recruited to the injured myocardium where they enhanced angiogenesis with resulting improvements in cardiac function [52]. *In vitro* models of AKI show migration of MSCs mediated by the SDF-1/CXCR4 pathway. In addition to this, the systemic transplantation of MSCs overexpressing CXCR4 was shown to accumulate at sites of AKI *in vivo* with associated improvement in renal function. This was correlated with an increase in SDF-1 expression in the injured tissue and was greater than the effect seen with unmodified MSCs [56]. In skeletal tissue, intravenously delivered MSCs are recruited by SDF-1 and participate in endochondral bone repair, supporting *in vitro* findings [55]. In addition, MSCs engineered to overexpress SDF-1 resulted in significantly augmented new bone formation at fracture sites in rat models [66].

As always, when considering the SDF-1/CXCR4 interaction in terms of MSC migration, it is important to consider the microenvironment in which MSCs function. Many of the *in vivo* models used are sites of hypoxic damage, while hypoxia is also a feature in inflammatory and neoplastic disorders [67–69]. It has previously been demonstrated that G-protein-coupled receptors, such as CXCR4, are regulated by hypoxia in the local environment [70]. Hypoxia has been shown to regulate CXCR4 expression in association with hypoxia inducible factor 1 (HIF-1) activation; hypoxia increases CXCR4 expression in mononuclear phagocytes, endothelial cells, and malignant cells, and levels remain high for a period after reoxygenation [71]. In malignancy, hypoxia upregulates CXCR4 expression and the likelihood of metastases in gastric cancer in an HIF-1-dependent manner [72], while CXCR4 is upregulated by several factors, including a deprivation of growth factors and hypoxia, in Ewing's sarcoma cells [73]. Other local factors that may influence SDF-1/CXCR4-mediated migration are local cytokines. Transforming growth factor (TGF)-β1, a cytokine with multiple functions, including regulation of cell proliferation and migration, downregulated expression of chemokine SDF-1 expression in the murine stromal cell line MS-5 [74]. In other studies, the migration of MSCs toward SDF-1 was increased after pretreatment of the cells with IL-1β and tumor necrosis factor-α (TNF-α), both potent proinflammatory cytokines found in conditions such as rheumatoid arthritis [75]. Interestingly, this did not appear to occur via an increase in CXCR4 expression or increased SDF-1 binding, and it has been hypothesized that proinflammatory cytokines may augment signal transduction without affecting receptor expression [75,76].

The SDF-1/CXCR4 pathway is well established as playing a central role in coordinating cell survival and migration in multiple cell types. It has now been established that this interaction also plays an important role in the migration of MSCs toward injured tissue both *in vitro* and *in vivo*. Understanding the role of cell culture and the local microenvironment will aid in increasing our ability to deliver site- and time-accurate MSCs and will also provide opportunities for cell modification to optimize targeted cell delivery.

While chemokines, and in particular the SDF1/CXCR4 interaction, have been implicated in the migration of MSCs to inflamed and hypoxic tissue, there remain unanswered questions; in particular, the reported expression rate of these chemokine receptors varies between published studies. This most likely reflects variations in *in vitro* culture conditions, isolation techniques, and time in culture [77–80]. In fact, long-term culture of MSCs results in a marked reduction in the expression of chemokine receptors and the loss of chemotactic responses to chemokines [22]. It is unlikely that activation of a single pathway is responsible for MSC migration, but rather that several chemokine axes play synergistic roles in the initiation and propagation of stromal cell migration. In addition to this there is evidence that integrins, toll-like receptors, matrix metalloproteinases (MMPs), and growth factors may also all play a part in this process.

Table 22.1 summarizes molecules influencing the migration of MSCs.

### 22.2.2 Integrins

Integrins are transmembrane receptors that mediate interactions between cells and between cells and the extracellular matrix. Integrin expression on MSCs has been implicated in the trafficking of MSCs to areas of cardiac ischemia [82]. Microarray technology and real-time (RT)-PCR were used to identify ligand–receptor pairs expressed in infarcted myocardium and BMSCs respectively. Receptors upregulated on MSCs that were thought may play a role in their recruitment included CXCR4, integrin α, and integrin β. These were then blocked by culture with the appropriate antibody. Receptor inhibition reduced adherence to fibronectin *in vitro*. However, only blockade of integrin 1β inhibited integration of locally delivered MSCs to infarcted myocardium [82].

### 22.2.3 Toll-like receptors

Another important player in MSC migration is the group of toll-like receptors (TLRs) which play a significant role in the pathology of a number of chronic inflammatory conditions, including rheumatoid arthritis and inflammatory bowel disease. It has been demonstrated that MSCs express TLRs at both the protein and RNA level with confirmation of TLR 1–6 expression on both human and murine MSCs isolated from either bone marrow or adipose tissue [89–92]. Additionally, as with chemokine receptors, their expression can be modulated by hypoxia with reported increases in TLR 1, 2, 5, and 9 under conditions of reduced

**Table 22.1** Factors influencing MSC migration.

| First author, year, ref | Molecules of interest | Findings |
|---|---|---|
| Honczarenko, 2006, [22] | CCR1, CCR7, CCR9, CXCR4, CXCR5, CXCR6 | All induced MSC migration with bell-shaped dose–response relationship |
| Xu, 2010, [27] | CCR2, CCR8 | May be activated to induce migration *in vitro* |
| Ringe, 2007, [32] | IL-8 | Increased MSC migration *in vitro* in dose-dependent manner |
| | MCP-1 | No *in vitro* migratory response |
| Boomsma, 2012, [39] | MCP-1 | Increased MSC migratory response |
| Belema-Bedada, 2008, [81] | MCP-1 | MCP-1 transgenic mice recruited MSCs *in vivo* |
| Schenk, 2007, [35] | MCP-3 (CCL7) | Increased migration to infarcted heart *in vivo* |
| Sordi, 2005, [38] | MIP-1α (CCL3) | Increased MSC migratory response |
| Wynn, 2004, [57] | SDF-1 (CXCL12) | Dose-dependent increase in MSC migration |
| Ryu, 2010, [58] | SDF-1 | Increased MSC migration *in vitro* |
| Ji, 2014, [30] | SDF-1 | *In vivo* migration to neural injury associated with increased SDF-1 expression |
| Liu, 2013, [56] | SDF-1 | MSCs overexpressing CXCR4 (receptor for SDF-1) accumulated at site of AKI |
| Ip, 2007, [82] | Integrin α, integrin β, SDF-1 | Receptor inhibition reduced adherence to fibronectin *in vitro*. Blockage of integrin 1β inhibited integration of MSCs to ischemic myocardium |
| Tomchuck, 2008, [83] | TLR3 | Stimulation increased MSC migration *in vitro* |
| Annabi 2001, [84] | MT1-MMP | Inhibition decreased MSC migration independently of MMP2 |
| De Becker, 2007, [85] | MMP2 | Inhibition of MMP2 reduced MSC migration *in vitro*. |
| | TIMP3 | Addition of TIMP3 reduced cell migration. |
| Ries, 2009, [86] | MMP2, MT1-MMP, TIMP2 | Gene knockdown impaired MSC migration. Silencing of TIMP1 increased migration |
| | TIMP1 | |
| Park, 2014, [87] | PLGF | Inhibition of PLGF receptor decreased MSC migration |
| Cipriani, 2013, [88] | PDGF | Augmented MSC migration *in vitro* |

CCR: C–C chemokine receptor type; CXCR: C–X–C chemokine receptor type; IL: interleukin; MCP: monocyte chemoattractant protein; MIP: macrophage inflammatory protein; SDF: stromal-cell-derived factor; TLR: toll-like receptor; MT: membrane type; MMP: matrix metalloproteinase; TIMP: tissue inhibitor of metalloproteinase; PLGF: placental growth factor, PDGF: platelet-derived growth factor.

oxygen concentration [93]. Stimulation of TLRs has been shown to activate numerous pathways, including signaling pathways involved in cell migration such as MAPK and PI3K [83,94]. In terms of migration, the role of TLRs remains unclear. The activation of TLR3 with several ligands was shown to increase human MSC migration in transwell chamber assays [83]. However, in wound healing models it was found that TLR 2 stimulation actually attenuated mouse MSC migration [95]. The roll of TLRs in stromal cell migration in inflammation warrants further study.

### 22.2.4 Matrix metalloproteinases

MMPs have also been implicated in MSC migration and in particular with the ability of MSCs to invade basement membrane and endothelial barriers. MMPs are proteolytic enzymes that are secreted as inactive proenzymes [96]. They are subdivided into groups based on their structure and ligand specificity. These groups are collagenases, stromelysins, matrilysins, gelatinases, and membranous-type MMPs. The gelatinases MMP2 and MMP9 are known to play a role in the migration and homing of leukocytes and hematopoietic progenitor cells [97–100]. Both human and murine marrow-derived MSCs have been shown to express MMP2 and MT1-MMP [84–86]. Both MMP2 mRNA expression and protein secretion have been confirmed [78,84]. In addition, murine MSCs and, human BMSCs and cord-blood-derived MSCs express MT1-MMP, while human BMSCs express tissue inhibitors of metalloproteinase (TIMPs) 1 and 2 [78,84,86].

These MMPs have been implicated in the migration of MSCs *in vitro*. Functional inhibition of MT1-MMP in murine MSCs decreases cell migration independently of MMP2 activation [84]. In human MSCs, inhibition of MMP2 with a neutralizing antibody inhibits, although does not completely block, MSC migration through basement membrane epithelium. A similar effect is seen when MSCs are transfected with MMP2 small interfering RNA (siRNA) [85]. The addition of recombinant TIMP3, a natural MMP inhibitor, to culture models led to a reduction in cell migration. Interestingly, TIMP3 is upregulated as MSCs

become more confluent and may be the mechanism behind the observed reduction in MSC migration as confluence increases [85]. In a similar study, the ability of MSCs to invade through reconstituted human basement membrane was blocked by a synthetic inhibitor of MMP. Gene knockdown of MMP2, MT1-MMP, and TIMP2 by RNA interference impaired migration, whereas the silencing of TIMP1 actually increased migration, suggesting that the TIMPs may have varying roles in MSC migration [86]. The relationship between MMPs, hypoxia, and cell confluence suggests that MMPs may play an important role in MSC migration, particularly the ability of MSCs not just to migrate to the site of injury but to cross endothelial basement membranes and reach damaged tissue [78,86].

### 22.2.5 Growth factors

It is also worth considering the role that growth factors may play in cell migration, with particular reference to placenta growth factor (PLGF) and platelet derived growth factor (PDGF). PLGF plays a role in endothelial cell proliferation and is upregulated in inflamed tissues, where it may play a role in the production of TNF-α, IL-1, IL-8, and MCP-1 [101–104]. Expression of the PLGF receptor Flt-1 has been found on MSCs and demonstrated to play a role in MSC migration *in vitro*. This was confirmed by reduction in migration after MSC preincubation with an anti-Flt-1 antibody [87].

PDGF has also been shown to be highly expressed at sites of inflammation and malignancy, and overexpression may enhance MSC migration to lung metastases *in vivo* [88,105]. PDGF-mediated augmentation of MSC migration *in vitro* can be abrogated by PDGF receptor inhibition [88]. PDGF receptors are expressed on MSCs and may represent a more potent MSC chemoattractant than SDF-1, but not after pretreatment with TNF-α [75,106].

## 22.3 The inflammatory milieu

As indicated above there are numerous mechanisms by which MSC migration may be initiated, with most of the work to date focusing on chemokines interacting with receptors on MSCs. There is much interest in how these data fit into the migration of MSCs under inflammatory conditions. There are several features of the inflammatory milieu that will be reviewed. Hypoxia, as outlined later, has been extensively studied and is important in terms not just of acute ischemic events but also as a feature of inflamed tissue. Other characteristic features to be considered include the presence of proinflammatory cytokines, the activation of the complement cascade, and the recruitment of macrophages.

### 22.3.1 Passive migration

Before reviewing these features it is worth considering that increased migration may simply be a passive process secondary to inherent features of inflammation, such as vasodilation and new vessel formation. However, given the increasing evidence of possible migration mechanisms, it is unlikely that this is the case, although it may facilitate homing *in vivo*.

### 22.3.2 Hypoxia

It is likely that the chemotactic function of MSCs is regulated by oxygen availability within the tissues. It is known that G-protein-coupled receptors, such as CXCR4, may be controlled by environmental signals, including tissue hypoxia, which plays a pathological role in inflammatory and neoplastic conditions [67–69,71]. HIF-1 is a central player in the hypoxia–CXCR4 pathway, increasing the expression of CXCR4 on monocytes, macrophages, and endothelial cells. HIF-1 activates gene transcription, resulting in adaptive responses to reduced oxygen availability. HIF-1-regulated genes include those that play a role in angiogenesis, vascular remodeling, and glucose metabolism [71]. In models of chronic obstructive pulmonary disease hypoxia led to an HIF-1-mediated increase in SDF-1, a factor implicated in MSC recruitment [107], while hypoxia in gastric cancer cells also upregulated CXCR4 [72]. In MSCs, temporary exposure to hypoxia induces a rapid increase in the nuclear translocation of HIF-1. However, the role of this in HIF-dependent kinase pathways in MSCs is not fully understood [108,109]. In MSCs, hypoxia-induced phosphorylation of MAPK has been reported as being both increased and downregulated [109–113]. Similar contradictions have been reported in terms of hypoxia-induced stress-activated protein kinase/c-Jun NH2 terminal kinase (SAPK/JNK) [110,112–114].

Specific studies in MSCs show that ischemic cerebral rat tissue induces a modest increase in human MSC migration *in vitro* compared with nonischemic tissue [115]. However, there are likely to be multiple factors involved in this, and what is perhaps more interesting is the effect of hypoxic culture conditions on MSC migration. Studies focusing on the effects of hypoxia on migration of MSCs generally suggest that short-term exposure to hypoxic culture conditions increases migration and may promote angiogenic activity.

One group has shown that 4 h of hypoxic culture conditions increased both the migration of MSCs *in vitro* and their ability to form three dimensional capillary-like structures. This effect appears to be mediated through increases in VEGF and MT1-MMP, the latter being an MMP implicated in MSC migration [84]. Another group showed that short-term (24 h) exposure to hypoxia increased the expression of CX3R1 (the receptor for fractalkine) and CXCR4 (the receptor for SDF-1) on human MSCs and was further increased with the addition of the iron chelator desferrioxamine, which stabilizes HIF-1α. Soluble fractalkine, also known as CX3CL1, potently chemoattracts T cells and monocytes, while the cell-bound chemokine promotes strong adhesion of leukocytes to activated endothelial cells, where it is primarily expressed.

Hypoxia also induced increased migration of these MSCs *in vitro* toward media both in the absence of chemokines and toward SDF-1α and fractalkine, ligands for CXCR4 and CX3R1

respectively [116]. More recent studies of cardiac progenitor cells also demonstrated CXCR4-mediated increases in migration secondary to hypoxic culture conditions [117]. *In vitro* scratch assays demonstrated that up to 10 h of hypoxia increased the migration of human UMSCs and that this effect was lost in the presence of siRNA for FAK. FAK plays a role in MAPK and HIF-1α pathways [110]. The *in vitro* scratch assay is an easy, low-cost, and well-developed method to measure cell migration *in vitro*. The basic steps involve creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells.

In addition, a hypoxia-induced increase in MSC migration in both wound healing and Boyden chamber assays was inhibited by blockers of ERK1/2 of c-Jun N-terminal kinase (JNK)1/2. An increase in these kinases was associated with an early increase in reactive oxygen species and a later HIF-1-dependent increase in VEGF. The migration of MSCs was decreased in the wound healing assay but not in the 6 h Boyden chamber assay after silencing of HIF-1α [112]. The Boyden chamber is a useful tool for studying cell migration. It consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Cells are seeded in the top of the insert in serum-free media, while serum or similar chemoattractants are placed in the well below. Migratory cells move through the pores toward the chemoattractant below and can be stained or quantified in a plate reader.

It is likely that the cellular responses of MSCs to hypoxia reflect a dynamic balance between pro- and antiapoptotic pathways [109]. However, there is clear evidence that short-term exposure to hypoxia may play a role in MSC migration in inflammation and could potentially be used as a tool to augment the migration of MSCs in a therapeutic setting.

### 22.3.3 Cytokines

When evaluating the migration of cells in an inflammatory environment it is important to consider the effect of proinflammatory cytokines on those cells (Table 22.2). Both TNF-α and IL-1β are proinflammatory cytokines that play an essential role in inflammation and may exert significant effects on cells exposed to them. It is known that MSCs are affected by the inflammatory milieu. There is a negative relationship between the degree of synovial inflammation and the chondrogenic and clonal capacities of human MSCs isolated from rheumatoid synovium [125]. Furthermore, *in vitro* studies have demonstrated an effect of proinflammatory cytokines on MSC differentiation and proliferation. TNF-α transgenic mice have decreased expression of osteoblast marker genes and osteogenesis and the proliferation of MSCs from C57BL/6 mice is suppressed by treatment with TNF-α or IL-1β [126,127]. However, it is important to be aware that not all MSCs behave in the same way: a direct comparison between MSCs from Balb/c mice (MHC $H2^d$) and FVB mice (MHC $H2^q$) isolated and cultured under identical conditions showed that the suppression of osteogenesis and adipogenesis by TNF-α or IL-1β was more pronounced in MSCs from Balb/c mice [124]. In addition to affecting differentiation there is evidence to suggest that proinflammatory cytokines can alter MSC proliferation *in vitro* and promote MSC-mediated tumor growth. The mechanism underlying this is not well understood but may involve upregulation of ubiquitin ligases or β-catenin-mediated upregulation of Wnt signaling [126,128,129].

It has been hypothesized that proinflammatory cytokines play a role in the migration of either endogenous MSCs or those delivered systemically to sites of inflammation. There is some suggestion that human MSCs migrate preferentially toward TNF-α [119], and most studies demonstrate a significant augmentation of *in vitro* migration after MSCs are pretreated with either TNF-α or IL-1β for 24 h. Adult MSCs were shown to migrate significantly more towards various chemokines, in particular MCP-1, RANTES, SDF-1, and macrophage-derived chemokines, after preincubation with TNF-α but not with IL-1β [75,119]. However, subsequent studies demonstrated that human BMSCs treated for 24 h with recombinant IL-1β had increased secretion of multiple chemokines involved in cell migration, including CCL5, CCL20, CXCL1, CXCL3, CXCL5, CXCL6, CXCL10, and CXCL11. This study also showed a strong pro-migratory role for IL-1β in *in vitro* transwell migration assays. With regard to nonhuman MSCs, TNF-α was shown to upregulate vascular cell adhesion molecule (VCAM)-1 expression on rat MSCs, and pretreatment of these MSCs with TNF-α increased the *in vitro* adhesion of MSCs to endothelial cells as measured with fluorescence microscopy. Furthermore, after intravenous delivery of fluorescently labeled MSCs in an ischemic muscle rat model, pretreated MSCs accumulated in greater numbers in the ischemic muscle after 24 h compared with MSCs not exposed to TNF-α [120].

MSCs isolated from both FVB and Balb/c mice also showed a marked increase in migration toward medium supplemented with serum *in vitro* [124]. *In vitro* and *in vivo* data by Zhang *et al.* exemplified the potential role of the inflammatory milieu on MSC recruitment in models of vascular damage. In a model of rat carotid artery injury, the overexpression of the signaling molecule SMAD3 led to an increase in MCP-1 production, a known chemoattractant for BMSCs. The phosphorylation of SMAD3 and the resultant increase in MCP-1 was thought to be mediated by TGF-β production in the smooth muscle of the injured vasculature [28].

While the upregulation of chemokines has been demonstrated, the exact mechanisms behind the increased migratory capacity of MSCs prestimulated with inflammatory cytokines remains unclear. Both TNF-α and IL-1β have known effects on the MAPK and PI3K pathways, both of which have been shown to mediate migration of chondrocytes and fibroblasts [130,131]. The role of the nuclear factor (NF)-κB has also come under scrutiny. NF-κB plays a central role in the coordination of immune responses in inflammatory conditions such as rheumatoid arthritis through transcription of cytokine genes, including

**Table 22.2** Summary of cytokine effects on MSC migration.

| First author, year, ref. | Cell type | Migration assay | Results |
|---|---|---|---|
| Ponte, 2007, [75] | Human BMSCs | Transwell assay, 8 μm pores, overnight | Pre-stimulation at 1 ng/mL for 24 h with TNF-α increased migration to SFM, SDF-1, MDC, RANTES. No increase in migration to GFs. Pre-stimulation at 1 ng/mL of IL-1β had no effect |
| | | | Pre-stimulation at 1 ng/mL of TNF-α increased MSC expression of CCR2, CCR3, CCR4, and membrane binding of RANTES |
| Böcker, 2008, [118] | Human BMSCs | Transwell polycarbonate membrane coated with ECM, 8 μm pores | Pre-stimulation at 30 ng/mL of TNF-α for 30 min increased expression of VCAM1, CD44, and MMP9 on MSCs, which was blocked by inhibition of NF-κB |
| | | | Increased migration towards 50 mg/mL TNF-α was reduced by blocking NF-κB |
| Baek, 2011, [119] | Human AMSCs | Transwell assay, polycarbonate membrane, 8 μm pores, 24 h | Prestimulation with 100 ng/mL of TNF-α increased migration toward SFM, PDGF-AB, TGF-β1, TNF-α, CXCL16, BCA-1, and RANTES |
| Xiao, 2012, [120] | Rat BMSC | 24-well culture plate coated with aortic rat endothelial cells | Pre-stimulation at 1–20 ng/mL of TNF-α for 24 h increased VCAM-1 expression by MSCs |
| | | | Pre-stimulation at 10 ng/mL of TNF-α increased adhesion to endothelial cells |
| | | *In vivo* rat hind limb ischemia | Pre-stimulation at 10 ng/mL TNF-α increased *in vivo* migration to ischemic muscle after intravenous delivery |
| Carrero, 2012, [121] | Human BMSCs | Transwell assay, 8 μm pores, 4 h | Pre-stimulation at 25 ng/mL of IL-1β increased expression of CCL5, CCL20, CXCL1 ICAMs, VCAM,and IL-8 on MSCs. Increased migration toward 25 ng/mL IL-1β, which was blocked by NF-κB |
| Fan, 2012, [122] | Human UCMSCs | Transwell assay, 8 μm pores, 12 h | Increased migration toward 10 ng/ml IL-1β, which was silenced by IL-1β RNA |
| | | Acute colitis *in vivo* | Pre-stimulation at 10 ng/mL of IL-1β increased migration to spleen, mesenteric lymph nodes, and colon *in vivo* after intravenous delivery |
| Xinaris, 2013, [123] | Mouse BMSCs | Scrape healing assay | Pre-stimulation at 20 ng/mL of IGF-1, 100 ng/mL of GDNF, and 100 ng/mL of TNF-α increased migration in scrape assay and toward cisplatin-injured cells and induced cytoskeletal changes in MSCs |
| | | Transwell assay, polycarbonate membrane, 8 μm pores, 24 h | Pre-stimulation with IGF-1 increased CXCR4 expression on MSCs |
| Sullivan 2014 [124] | Mouse BMSCs | Transwell assay, 8 μm pores, 18 h | Pre-stimulation at 50 ng/mL of TNF-α or IL-1β increased migration to 10% serum. Not affected by NF-κB inhibition |

BMSC: bone-marrow-derived mesenchymal stromal cell; AMSC: adipose-tissue-derived MSC; UMSC: umbilical-cord-derived MSC; TNF-α: tumor necrosis factor-α; SFM: serum-free media; SDF-1: stroma-derived factor 1; MDC: macrophage-derived chemokine; GF: growth factor; IL: interleukin; CCR: C–C chemokine receptor type; ECM: extracellular matrix; VCAM: vascular cell adhesion molecule; CD44: cluster of differentiation 44; MMP: matrix metalloproteinase; NF-κB: nuclear factor kappa B; PDGF: platelet-derived growth factor; CXCL: C–X–C motif chemokine; BCA: B cell attracting chemokine; ICAM: intercellular adhesion molecule; IGF: insulin-like growth factor; GDNF: glial-derived neurotrophic factor; CXCR: C–X–C chemokine receptor type; RANTES: regulation on activation, normal T cell expressed and secreted (also known as CCL5).

TNF-α and IL-1β. This pathway also plays a role in the regulation of genes involved in cell proliferation, migration, and differentiation and may induce the expression of adhesion molecules, enhance vascular permeability, and recruit inflammatory cells [132]. Inhibition of the NF-κB pathway has been shown to reverse the pro-migratory effects of TNF-α on human MSCs [118,121]. Another study using UMSCs demonstrated an increase in migration in response to *Staphylococcus aureus*-infected epithelial cells, which expressed high levels of TNF-α. This effect was abrogated by using siRNA to knock down the p65 subunit of NF-κB [133]. However, this was not the case for MSCs derived from mouse bone marrow. The inhibition of NF-κB, either by transduction of MSCs with an adenoviral vector expressing a super-repressor of NF-κB or by addition of the anti-inflammatory agent curcumin to the culture medium, had no effect on the augmentation of murine Balb/c MSC migration after stimulation with TNF-α *in vitro* [124].

Table 22.2 summarizes the effects of cytokines on MSC migration.

### 22.3.4 Complement

The complement system is a complex cascade that can be activated either by the classical pathway, which is dependent on an antigen–antibody reaction, or by an alternative pathway,

which is usually mediated by bacterial endotoxins or through the binding of lectin to mannose on the surface of pathogens. All three pathways culminate in a final common pathway with production of bioactive peptides such as C3a and C5a [134]. Components of the complement system, including C1, which initiates the classical pathway, have been shown to play a role in the chemoattraction of several inflammatory cell types, including dendritic cells, mast cells, eosinophils, and neutrophils [135–139]. Therefore, it is possible that components of the complement system play a role in the chemoattraction of MSCs to sites of inflammation. It was shown in one study that both C3a and C5a are chemoattractants for human BMSCs that express C3a and C5a receptors and that this may be mediated by prolonged phosphorylation of the ERK1/2 pathway [140]. More recent studies looked at the role of C1q, a subcomponent of the C1 complex, in MSC recruitment. C1q was shown to chemoattract MSCs in a dose-dependent manner through gelatin and, to a lesser extent, through Matrigel *in vitro*. MSCs were found to express C1q receptors. However, the effects of C1q appear to be multifactorial, and findings included an increase in MSC CXCR4 expression with increased chemotaxis to SDF-1, an increase in proMMP2 secretion, and prolonged ERK1/2 phosphorylation in C1q-stimulated MSCs [141].

The effect of complement on other MSC functions has also been investigated. Studies have suggested that C3a and C5a may protect MSCs from oxidative damage since pretreatment of MSCs with these complement components decreased cell death in MSCs exposed to $H_2O_2$ *in vitro* [140]. Additionally, MSCs express CD46, CD55, and CD59, which confer protection from complement-mediated lysis [142]. However, Li and Lin suggested that these intrinsic cell surface regulators are insufficient protection against complement-mediated cytotoxicity and that the membrane attack complex, the common endpoint of complement activation, directly injures MSCs. This effect was found to be more pronounced after *in vivo* delivery of allogeneic murine MSCs compared with delivery of autologous murine MSCs [143]. Whether complement activation in the complicated setting of inflammation *in vivo* is harmful to MSCs or stimulates their recruitment is currently unknown.

### 22.3.5 Macrophages

Macrophages play a central role in the microenvironment of tumors and wound healing. They likely sense tumors as a type of wound, given the increased angiogenesis and the leukocyte infiltration characteristic of many tumors. In tumors they can further stimulate angiogenesis and promote metastatic spread of the disease [144–147]. Macrophages may play an important role in the attraction of MSCs to sites of injury. In a study by Anton *et al.* MSCs were cultured with macrophage-conditioned medium. This resulted in a change in MSC gene expression into a proinflammatory phenotype with increased gene expression of several factors involved in MSC migration, including CCL2, CXCL12, and IL-8. Interestingly, however, CXCL12 and IL-8 protein secretion did not increase [145]. Macrophage-conditioned medium increased MSC migration *in vitro*. This was mediated by CCL5, CCL2, and IL-8 (chemotactic factors secreted by macrophages) and was dependent on activation of a JNK pathway. There was some reduction in MSC migration by blocking CCL5 and IL-8, but not CCL2. This suggests that these factors alone may not be essential pathways for MSC migration but may play role in combination with other mechanisms.

## 22.4 Mesenchymal stromal cell extravasation

There have been numerous studies looking at the factors present in the inflammatory environment that may influence and augment MSC migration. Less is known about the physical behavior of MSCs that allows them to migrate through endothelial cells, a process known as extravasation. This is critical in permitting MSCs to leave the circulation and access injured tissue. The process of leukocyte extravasation is well understood and involves an active process of rolling, endothelial activation, lateral migration over endothelium, and the use of dynamic cytoskeletal protrusions such as pseudopods and lamellipodia to cross through discrete gaps in the endothelium [148,149]. MSCs have been shown previously to roll and adhere. However, the process of MSC integration in endothelium appears to differ from that of leukocytes [150–153]. High-resolution confocal microscopy and dynamic live-cell imaging was used by Teo *et al.* to characterize the extravasation of human BMSCs through endothelial cells activated with TNF-α [154]. MSCs were seen to adhere to the endothelial monolayer before becoming incorporated into a transmigratory cup formed by microvilli-like endothelial projections. MSCs did not spread like leukocytes but formed bulbous protrusions that facilitated either paracellular or transcellular endothelial integration and then subendothelial spreading. This occurred over a much longer timespan than leukocytes, taking up to 2 h compared with several minutes [154]. These data indicated that MSC migration shares some features with that of leukocytes. However, the nonapoptotic blebbing seen is similar to that seen on embryonic germ cells and metastatic tumor cells [155–157]. The role of MSCs in inflammation was also suggested when MSC endothelial transmigration was observed across endothelial barriers activated by the potent proinflammatory cytokine TNF-α. *In vitro* static models can never fully predict *in vivo* behavior and shear flow, which, along with the condition of the vascular bed in actively inflamed tissues, will almost certainly impact on MSC behavior in the setting of inflammation.

## 22.5 *In vivo* migration

### 22.5.1 *In vivo* migration studies

While there are numerous studies exploring the capacity of MSCs to migrate *in vitro*, the ultimate goal in terms of therapeutics is

the homing of systemically delivered MSCs to sites of injury *in vivo*. Over the last decade there have been a large number of reports of MSC homing in numerous disease models, including malignancy, ischemia, brain injury, pulmonary fibrosis, arthritis, and AKI. Various methodologies have been used to track MSCs, including immunohistochemistry, PCR, *in situ* hybridization, and live imaging.

Some early MSC homing studies were carried out in primates after total body irradiation [158]. Intravenously delivered autologous and allogeneic MSCs were shown to distribute widely to the gastrointestinal tract, lung, liver, and skin [158]. Later studies in NOD/SCID mice given total body irradiation demonstrated increased engraftment of human BMSCs to multiple organs compared with nonirradiated mice. Migration was further increased in areas of local tissue irradiation [159].

The development of novel therapies for use in the treatment of malignancy remains a high priority; not surprisingly, numerous studies have been carried out in an attempt to confirm the homing of systemically delivered MSCs in *in vivo* tumor models. Human BMSCs were delivered systemically to SCID mice after injection of cells from a melanoma tumor cell line. Immunohistochemistry was used to identify the X-Gal-labeled cells and after three intravenous doses cell colonies were demonstrated in the tumor stroma. When modified to express IFN-β, a reduction in tumor size after MSC administration was noted [160]. Several studies have examined the migration of MSCs in murine glioma models. Fluorescently labeled human BMSCs were delivered intra-arterially using either the carotid or intracerebral artery 10 days after glioma tumor implantation. In one study tissue was examined 1 and 7 days after the infusion of the MSCs. Fluorescent imaging identified MSCs only at the tumor sites [161]. Further glioma studies in mice examined the migration of human BMSCs modified with adenovirus for luciferase expression to high and low PDGF-expressing human glioma xenografts. After intra-arterial injection, MSCs were shown to migrate to the malignant tissue with increased engraftment in PDGF high-secreting tumors, and this correlated with *in vitro* data [105]. MSC homing has also been studied in mice with human breast cancer xenografts and intravenously delivered breast cancer cell lines to mimic metastatic disease [29]. Fluorescently labeled cells were delivered either subcutaneously or intravenously. After subcutaneous delivery, MSCs were identified at the tumor border, while intravenous delivery of cells in models of metastatic disease resulted in migration to tumor sites and lining blood vessels. By correlation with *in vitro* studies, MCP-1 has been implicated as a potential mechanism driving migratory events [29]. More recently, both human BMSCs and human umbilical cord perivascular cells (HUCPVCs) were shown to migrate to subcutaneous hepatocellular carcinoma implants in mouse models using histology and fluorescent microscopy. Cells were also found in the lung, liver, and spleen after intravenous delivery. A higher number of HUCPVCs engrafted in the tumor compared with BMSCs [162]. Other recent studies in malignancy include the demonstration of both human MSCs and murine MSCs homing to the site of mesothelioma, a malignancy of the lung pleura, after systemic delivery [163] and the successful treatment of gastric cancer xenografts in nude BALB/c mice after systemically delivered human BMSCs expressing NK4, in which $GFP^+$ MSCs were detected at the tumor site by immunohistochemistry 28 days post-systemic injection [164]. (NK4 is an inhibitor of angiogenesis as well as an HGF antagonist.)

Many studies have demonstrated MSC homing in models of ischemic diseases, particularly cerebral and cardiac ischemia. Lac-Z-transfected human BMSCs were delivered intravenously to rats after occlusion of the middle cerebral artery (MCA). The MCA is one of the three major paired arteries that supply blood to the cerebrum. The MCA arises from the internal carotid and continues into the lateral sulcus, where it then branches and projects to many parts of the lateral cerebral cortex. It also supplies blood to the anterior temporal lobes and the insular cortices (Figures 22.1 and 22.2).

Immunohistochemistry in this study demonstrated Lac-Z-positive cells in the cerebral infarct area after both single and multiple injections of MSCs and which were associated with neovascularization and improved outcomes [165]. Another study described the delivery of fluorescently labeled human MSCs to neonatal mice with hypoxic ischemic brain injury [166]. After intranasal delivery the cells were found in the sensorimotor and epithalamic region around pathological cysts, with no cells found in the contralateral (opposite) normal hemisphere. Treated mice had improved motor behavior and a decrease in the size of the hypoxia-induced lesions [166]; and see Chapter 16.

The role of MSCs in the treatment of acute cardiac ischemia and their potential to home to infarcted myocardium has also been extensively investigated. $^{99m}$Tc- or BrdU-labeled rat MSCs were delivered either intravenously or directly into the left ventricle of the heart 2, 10, or 14 days after ligation of the left anterior descending (LAD) coronary artery, which supplies the left ventricle and which is most commonly obstructed in people with an acute myocardial infarct (Figure 22.3).

Cells delivered intravenously were found predominantly in the lungs, with only a small number reaching the infarct zone. The MSCs injected directly into the myocardium integrated into the infarct zone [167]. Further studies in a similar model confirmed the migration of MSCs to infarcted myocardium after intravenous delivery using immunohistochemical staining. This was associated with a reduction in infarct size compared with mice that received vehicle control [168]. Subsequently, both fluorescent *in situ* hybridization (FISH) and confocal microscopy have been used to confirm the migration of intravenously delivered rat BMSCs to infarcted areas within 24 h of, and up to 8 weeks after, ligation of the LAD coronary artery [35,169].

*In vivo* imaging has also been carried out in canine models of myocardial ischemia [170]. Allogeneic MSCs were co-labeled with a radiotracer and a magnetic resonance contrast agent and delivered intravenously to dogs after occlusion of the LAD coronary artery. High-sensitivity single-photon emission computed tomography (SPECT) and magnetic resonance imaging

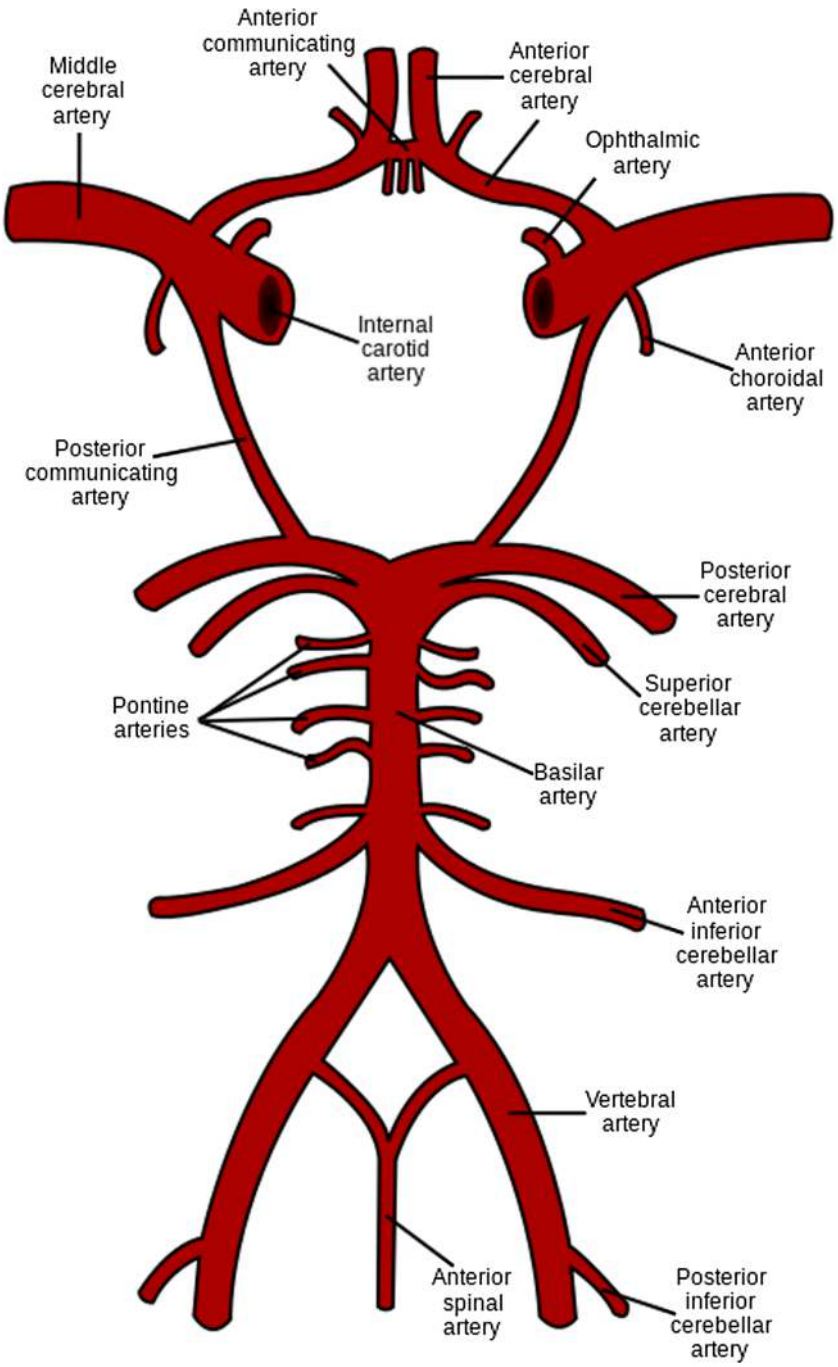



**Figure 22.1** The arterial supply to the brain, including that of the MCA. *Source:* http://commons.wikimedia.org/wiki/File:Circle_of_Willis_en.svg#/media/File:Circle_of_Willis_en.svg.

(MRI) were used to track the fate of these cells *in vivo*. After initial infusion the cells migrated to the lungs and then to other nontarget organs. However, within 24 h, focal and diffuse uptake of MSCs was seen in the infarcted myocardium using SPECT but not MRI [170].

As well as cerebral and myocardial ischemia, there have been studies of MSC delivery in hepatic ischemic–reperfusion injury models [171]. Murine MSCs were delivered intravenously and tracked either with immunohistochemistry using Lac-Z or by use of a radioactive label. While MSCs were initially found in the lung and subsequently in the liver at 24 h after injection, there was no increase in migration to the injured liver compared with controls [171]. Therefore, while there is evidence to support the migration of exogenously delivered MSCs to ischemic tissues, it is important to remember the limitations inherent in the techniques used to identify them, as well as possible differences in the degree or type of chemoattraction occurring in ischemic injury in different organs.

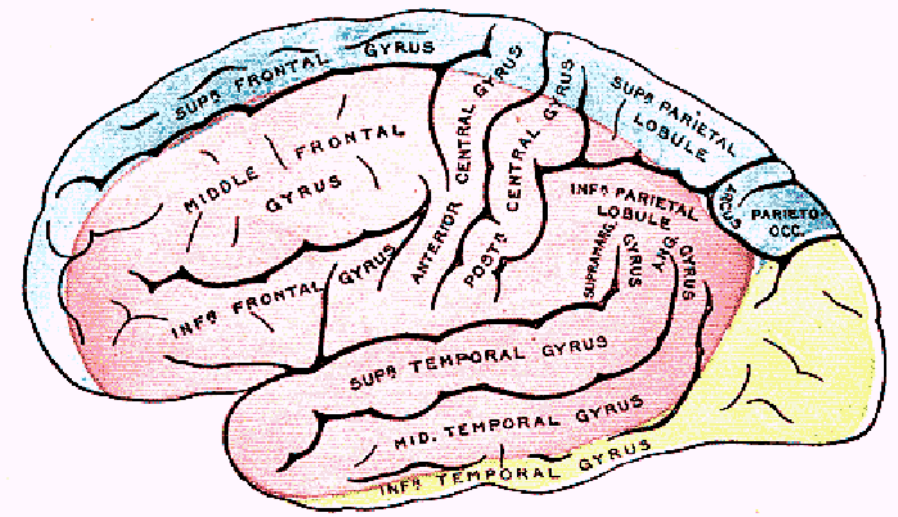



**Figure 22.2** Outer surface of a human cerebral hemisphere showing areas supplied by the cerebral arteries (see text). *Source: Gray's Anatomy*, bartleby.com.

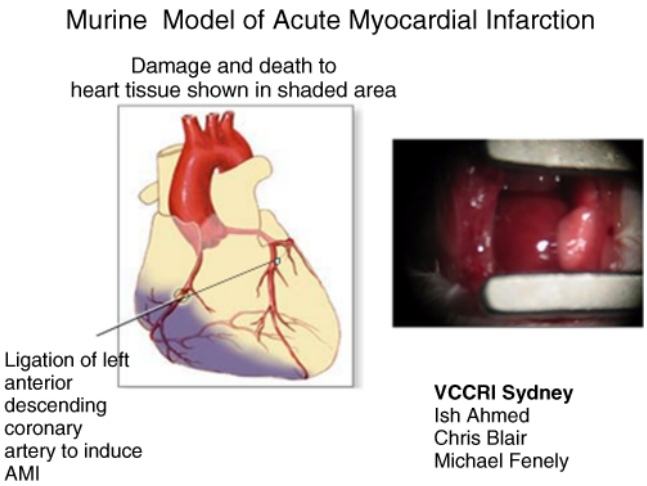



**Figure 22.3** Occlusion of the LAD coronary artery in a mouse at open thoracotomy under general anesthesia by ligation of the artery to induce an acute myocardial infarction (AMI) in the wall of the left ventricle.

Another commonly used model for studying the homing of MSCs is that of AKI. Various toxins have been used, including administration of glycerol or cisplatin [172–174]. Both murine and human MSCs have been shown to migrate to the renal cortex, peritubular cells, and tubular epithelial lining after intravenous delivery of MSCs, with associated amelioration of disease and improved survival [172–174].

In inflammatory arthritis studies there have been conflicting results. The intraperitoneal delivery of allogeneic MSCs to mice with collagen-induced arthritis (CIA) was not shown to result in homing to the joints, but rather initial localization in the peritoneum with subsequent migration to the spleen [12]. However, in a subsequent study using the intravenous route of delivery, PCR for green fluorescent protein (GFP) was used to distinguish $GFP^-$ MSCs from $GFP^+$ transgenic murine MSCs in the paws and knees of mice with CIA. In this study, GFP was also identified, as expected, in the lungs and liver of both arthritic and control mice [175].

Nonischemic cerebral injury has been another target of MSC administration, including experimental autoimmune encephalitis (EAE), hypoglossal (12th cranial) nerve injury, and models of traumatic brain injury [30,176–178]. In EAE and brain injury models, MSCs were found to penetrate the cord and the subarachnoid space (in the EAE model) as well as the boundary zone of traumatic brain lesions. In addition, the migration of MSCs reduced inflammation and demyelination and improved functional outcome [176–178]. In the model of hypoglossal nerve injury, rat MSCs labeled with carboxyfluorescein *N*-hydroxysuccinimidyl ester dye were delivered to the lateral ventricle of rats at the time of injury [30]; significantly more MSCs were found at the injured nerve site compared with the contralateral control at 1 week, which appeared to be related to fractalkine and SDF-1 expression at the site of nerve damage.

### 22.5.2 Controversies surrounding *in vivo* migration

Studies of *in vivo* models using exogenously delivered MSCs are shown in Table 22.3.

Preclinical models have included muscle injury, pulmonary fibrosis, and wound healing. What is immediately obvious is the large variety of cell types, injury models, delivery routes, and tracking methods used. This makes it difficult to compare studies. MSCs have been derived from bone marrow, adipose tissue, and umbilical cord in numerous species, including rat, mouse, dog, and human. There is variation with regard to the site of isolation and the media and incubation methods used. These variables have profound effects on the migratory potential of cells, as has been seen with hypoxia studies outlined earlier.

For initial consideration is the term MSC itself. The Mesenchymal and Tissue Stromal Cell Committee of the International Society for Cellular Therapy published criteria for defining MSCs in 2006. These required that cells be plastic-adherent, positive for

**Table 22.3** Summary of *in vivo* migration studies.

| First author, year, ref. | MSC type | Disease model | Route | Tracking method | Migration |
|---|---|---|---|---|---|
| Barbash, 2003, [167] | Rat BMSCs | Rat LAD ligation To induce AMI | IV or intraventricular | Gamma camera, immunohistochemistry | IV cells mostly lodged in lungs Intraventricular cells detected in infarct zone. |
| De Bari, 2003, [179] | Human synovial fluid MSCs | Nude mouse muscle injury | Local | ISH, immunohistochemistry | 1 week: detected in mononuclear cells 2 weeks: detected in myofibers |
| Devine, 2003, [158] | Primate BMSCs | Total body irradiation | IV | RT-PCR | Wide tissue distribution – gastrointestinal tract, kidney, lung, liver, skin |
| Ortiz, 2003, [180] | Mouse BMSCs | Lung injury | IV | RT-PCR, FISH | >20% increase in male DNA in injured lungs Engrafted cells localized to injury |
| Rombouts, 2003, [181] | Mouse BMSCs | Irradiation | IV | CFU-F assay | Uncultured cells >50% in marrow, 7% spleen After 24 h culture: 10% in marrow After 48 h culture: not detected in marrow |
| Morigi, 2004, [174] | Human BMSCs | Acute kidney injury | IV | ISH, immunohistochemistry | MSCs localized to tubular epithelial lining |
| Nagaya, 2004, [168] | Rat BMSCs | Coronary artery ligation to induce AMI | IV | Immunohistochemistry | Cells detected in infarcted myocardium |
| Studeny, 2004, [160] | Human BMSCs | SCID mouse with lung cancer | IV | Histochemistry | Cells detected in lung tumor stroma |
| Ji, 2004, [30] | Rat BMSCs | Hypoglossal nerve injury | IV | Confocal microscopy | Significantly more rat MSCs at left HN compared with right. Greatest difference after 1 week |
| Kraitchman, 2005, [170] | Canine BMSCs | LAD occlusion to induce AMI | IV | MRI, SPECT | Initial uptake in lungs. Redistributed to liver, kidney, spleen after 24 h. Engraftment in infarcted heart at 1–7 days |
| Mahmood, 2005, [178] | Human BMSCs | Brain injury | IV | Immunohistochemistry | MSCs present in lesion boundary zone |
| Nakamizo, 2005, [161] | Human BMSCs | Mouse with glioma | IA | Fluorescent microscopy | Detection of cell marker at tumor sites only |
| Zappia, 2005, [177] | Mouse BMSCs | Mouse EAE | IV | Immunohistochemistry | Detection in spleen and nodes after 24 h, in subarachnoid space after 40 days |
| François, 2006, [159] | Human BMSCs | NOD/SCID TBI | IV | PCR and immunohistology | Increased engraftment to organs after TBI and locally to irradiated tissue |
| Horita, 2006, [182] | Human BMSCs | Rat MCAO | IV | Immunohistochemistry | Detected at ischemic penumbra |
| Jiang, 2006, [169] | Rat BMSCs | Rat LAD ligation to induce AMI | IV | FISH | Detected in infarct or border zone |
| Schenk, 2007, [35] | Rat BMSCs | Rat LAD ligation | IV | Confocal microscopy | Transient migration to infarct zone within 24 h. MSCs in infarct zone with overrepression of MCP-3 and after serial infusions |
| Dwyer, 2007, [29] | Human BMSCs | Mouse: breast cancer | SC, IV | Florescent microscopy | SC: cells at tumor border. IV: detected in patches throughout tumor |
| Augello, 2007, [12] | Mouse BMSCs | CIA | IP | Immunohistochemistry | Localized to peritoneum and spleen only |
| Kanehira, 2007, [183] | Mouse BMSCs | Mice: metastatic tumor | IV | Immunohistochemistry | Detected in tumor tissue in lung |
| Herrera, 2007, [172] | Mouse BMSCs | Acute kidney injury | IV | Immunohistochemistry | In renal cortex after 24 h. A few CD44-negative cells migrated |

**Table 22.3** (*Continued*)

| First author, year, ref. | MSC type | Disease model | Route | Tracking method | Migration |
|---|---|---|---|---|---|
| Sasaki, 2008, [184] | Mouse BMSCs | Mouse punch biopsy wound | IV | Flow cytometry | MSCs in skin wound 3 days after injection. Improved wound healing |
| Omori, 2008, [165] | Human BMSCs | Rat MCAO | IV | Immunohistochemistry | Cells detected in cerebral infarct area |
| Morigi, 2008, [173] | Human BMSCs | NOD/SCID mouse AKI | IV | Immunohistochemistry | Cells located in tubular epithelium and peritubular cells |
| Loebinger, 2009, [185] | Human BMSCs | NOD/SCID mouse: IV xenograft – lung metastases | IV | MRI | MSCs integrated in pulmonary metastases |
| Constantin, 2009, [176] | Mouse AMSCs | Mouse EAE | IV | Flow cytometry and BLI | Control mice: cells in nodes and spleen EAE mice: intralesional MSCs |
| Hata, 2010, [105] | Human BMSCs | Mouse: high and low PDGF-expressing human glioma xenograft | IA | Immunohistochemistry and BLI | MSCs migrated to glioma. Increased in PDGF high secreting tumors |
| Eggenhofer, 2012, [171] | Mouse BMSCs | Mouse ischemia reperfusion liver injury | IV | Immunohistochemistry | MSc in lung after 1 h and in liver within 24 h. No increased migration in injury model |
| Donega, 2013, [166] | Human BMSCs | Mouse: hypoxic ischemic brain injury | Intranasal | Immunohistochemistry | MSCs detected in sensorimotor and epithalamic region of affected hemisphere |
| Sullivan, 2013, [175] | Mouse BMSCs | CIA | IV | RT-PCR | Detected in lung and liver control group Detected in joints of CIA mice at 5 and 14 days |
| Bayo, 2014, [162] | Human BMSCs and UC perivascular cells | Mouse hepatocellular carcinoma | IV | Histology | Both cell types detected in lung, liver, spleen, and tumor |
| Zhu, 2014, [164] | Human BMSCs | Nude mouse: gastric cancer xenograft | IV | Immunohistochemistry | GFP detected in tumor xenografts |
| Lathrop, 2014, [163] | Mouse and human BMSCs | Mouse: mesothelioma xenograft | IV or IP | Immunofluorescent staining | Cells delivered by either route localized to tumor |

AMI: acute myocardial infarction; BMSC: bone-marrow-derived mesenchymal stromal cell; AMSC: adipose-tissue-derived MSC; UC: umbilical cord; IV: intravenous; IA: intra-arterial; ISH: *in situ* hybridization; IP: intraperitoneal; SC: subcutaneous; FISH: fluorescent *in situ* hybridization; RT-PCR: real-time polymerase chain reaction; MRI: magnetic resonance imaging; SPECT: single photon emission computed tomography; LAD: left anterior coronary artery; AKI: acute kidney injury; MCAO: middle cerebral artery occlusion; EAE: experimental autoimmune encephalitis; CIA: collagen-induced arthritis; HCC: hepatocellular carcinoma; SCID: severe combined immunodeficiency; NOD: nonobese diabetic; PDGF: platelet-derived growth factor; BLI: bioluminescence imaging; MCP: monocyte chemoattractant protein; TBI: total body irradiation; HN: hypoglossal nerve (12th cranial nerve).

CD105, CD73, and CD90 cell surface markers, and negative for CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR cell surface markers. Cells had to be capable of trilineage mesodermal differentiation, specifically chondrogenesis, osteogenesis, and adipogenesis [18]. It is likely that prior to the introduction of these criteria that the term MSC was applied loosely to bone marrow cells.

After initial isolation the time in culture at which cells are used for studies may play a significant role in their migratory capacity. Rombouts and Ploemacher published a study looking at syngeneic MSCs delivered to C57BL/6 mice after sublethal irradiation [181]. Delivery of uncultured cells resulted in recovery of 55–65% of injected colony-forming units–fibroblast from the bone marrow. After culture for 24 h only 10% were recovered, while this decreased to 0% after 48 h of culture. There are several reasons why time in culture may influence homing potential. It may partly be due to the variability of cell surface receptor expression, with published studies demonstrating changes in SDF-1 expression over time on culture-expanded MSCs. In one report, culture-expanded BMSCs were studied to passage 16: at higher passages there was a loss of cell surface expression of all chemokine receptors studied (CCR1, CCR7, CCR9, CXCR4, CXCR5, CXCR6) and an associated lack of chemotactic response to chemokines [22]. There was also a

loss in MSC expression of adhesion molecules, an increase in apoptosis, and slowing of cell growth. Of interest is the fact that not all cells lost their receptor expression at the same rate: a major subpopulation was completely negative while some retained high levels of expression [22]. In addition, it has been shown that expression of CD105, CD146, and CD271 are upregulated in culture. In addition to this, MSC morphology changes with subsequent passages, becoming larger and more spindle shaped with diameters larger that of pulmonary microcapillaries [186–190]. Clearly, this may impact on *in vivo* studies in which passage number has not always been reported. For those reported there is a large variation in passage (P) number, ranging from P2 to P23 [35,159,162,173,176].

In addition to passage number, the degree of confluency of MSCs before harvesting has been shown to influence migration. This is harder to report accurately. In high-confluence cell cultures there may be an increase in the production of the natural MMP inhibitor TIMP3; this results in a decrease in transendothelial migration and subsequent migration to target organs [85].

Finally, the age of the animal from which the cells were harvested may also play a role in MSC migration. It has been suggested that the age of MSC donors may affect differentiation potential and proliferative capacity, although this is controversial [190–195]. Reports have suggested that older mouse donors may provide MSCs with lower expression of cytokine and chemokine receptors, thus compromising their ability to migrate to sites of injury [196].

The route of administration may also play an important role in the migration of cells to injured tissue. In some clinical settings systemic delivery by the intravenous, and less so the intra-arterial, route is preferred to local injection. Particularly problematic after intravenous cell delivery is the trapping of MSCs in the lung, with only very small percentages subsequently managing to reach target organs (Figure 22.4).

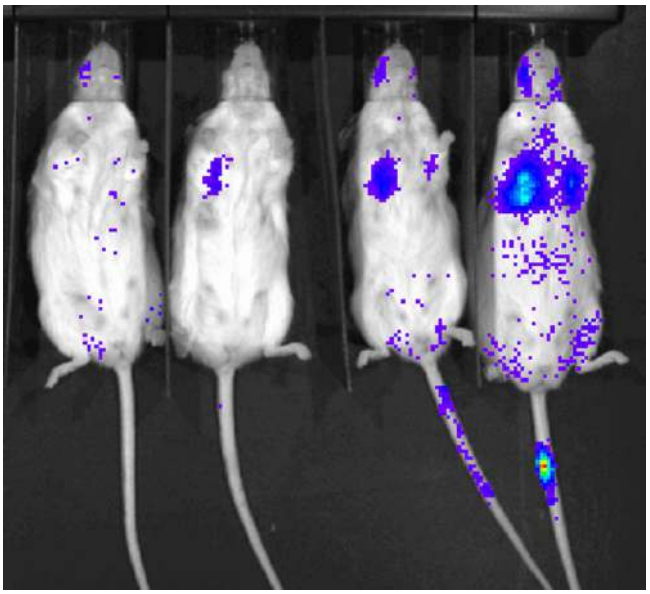


**Figure 22.4** Luciferase-positive murine MSCs injected intravenously lodge in the lungs for at least 3 days, presumably because their size prevents them from flowing freely through the pulmonary capillary bed, which is the first network of capillaries that they encounter after intravenous injection.

Migration to liver, spleen, kidney, marrow, or injured tissues is slow, and this may affect the timing of cell delivery [197,198]. However, when a small subcutaneous skin wound was made on the right side of the abdomen prior to the intravenous injection of luciferase-positive MSCs, a significant number of the injected cells passed through the lungs and homed preferentially to the wound site (Figure 22.5).

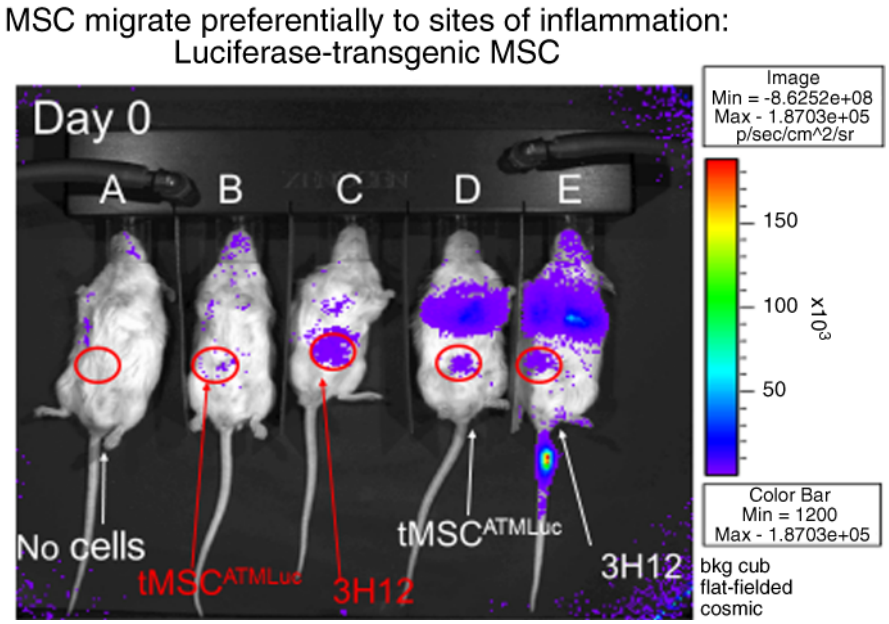



**Figure 22.5** Luciferase-positive MSCs injected intravenously in a mouse in which a subcutaneous incision had been made on the right side of the mouse's abdomen prior to injection of the MSCs. No wound was made on the left side of the mouse's abdomen. While the majority of MSCs were retained in the lungs, some MSCs flowed through the lungs and homed preferentially to the site of the skin wound on the right side of the mouse's abdomen. (See color plate section.)

Compounding the problem, however, is the short half-life of MSCs, with many cells reported to be apoptotic 12 h after injection [199,200]. Potential strategies that have been used to augment MSC migration out of the lung include co-infusion with heparin or with sodium nitroprusside treatment [190,198,200].

There are also specific challenges associated with MSC migration after intra-arterial delivery, most notably that of plugging in the microvasculature leading to obstruction of flow at a precapillary level with ensuing ischemia and loss of the injected cells [201]. This is a mechanical obstruction due to the relative size of MSCs compared with the lumen of the microvasculature, with a few surviving cells spread out on the luminal surface of small arteries and then localizing in the perivascular niche within 72 h [201]. Intravital fluorescent microscopy of the cremaster muscle (the muscle that covers the testis and the spermatic cord in males) of SCID mice after injection of human AMSCs into the abdominal aorta showed similar results. Decreased blood velocity was noted in a cell-density-dependent manner until the stoppage of blood flow in the cremaster microcirculation [202]. An interesting consideration in this regard are published results that the size of MSCs may actually increase with time in culture, further decreasing the migratory potential of high-passage cells [203,204].

Along with the route of administration, the number and frequency of MSCs infused may be critical in facilitating the desired migratory response. The idea that higher numbers of infused cells will result in higher numbers of engrafted cells does not necessarily hold true. In general, in rat and mouse models of disease, MSC doses between $1 \times 10^5$ and $1 \times 10^6$ seem to be optimal and are commonly used doses in most models outlined [162,164,165,175]. In models of brain injury, no additional engraftment was seen when the number of infused cells was increased from $1 \times 10^6$ to $3 \times 10^6$ in a rat model of brain ischemia [205]. It is possible that a plateau may be reached after a certain number of cells are infused, though there are multiple factors that may affect this further, as outlined earlier.

The MSC recipient animal should also be considered. The disease models studied represent a large array of diverse pathologies, including ischemia, malignancy, inflammation, drug-induced injury, and trauma. The degree to which inflammation is localized to a specific tissue, such as in a tumor xenograft, compared with the systemic inflammation associated with CIA is likely to impact on the behavior of the administered MSCs, though it is unclear to what extent this affects migration. Additionally, it is worth noting that many of the injury models use severely immunocompromised host animals, and the effect of this on migration and homing is not understood [158,159,173,206].

One of the final but most important considerations in terms of *in vivo* homing studies is the methods used to detect the administered MSCs. Methods published in the literature include fluorescent protein labeling, transduction of MSCs with reporter genes, detection of human genes in animal recipients, sex-linked gene detection, histology, immunohistochemistry, RT-PCR, and FISH [158,161,165,168,173,174,178,180,182,197]. The main limitation of these techniques is that they provide a static measurement without any real-time information on the fate of infused cells. Additionally, the detection of a label does not necessarily indicate live cell engraftment but may represent a macrophage that has phagocytosed a labeled MSC [171]. The role of dynamic *in vivo* imaging will be discussed later in this chapter. It is worth remembering that the methods generally employed to assess homing efficiency are often compared with controls or with nondiseased tissue.

### 22.5.3 Real-time *in vivo* imaging

The majority of studies investigating *in vivo* migration of MSCs to sites of inflammation, ischemia, and malignancy rely on *ex vivo* immunohistochemistry, histology, and PCR techniques. While these methods can provide valuable data they lack sufficient spatial and temporal information. Additionally, disruption of the target organ is necessary, and sequential time points are difficult to assess. Work is ongoing to harness modern imaging techniques to more fully understand the dynamics of *in vivo* cell biology, allowing multiple tissues to be examined at multiple time points. These include MRI, SPECT, positron emission tomography (PET), and bioluminescence imaging (BLI). In view of the increasing use of these modalities and their implications for ongoing studies in this field, they will be briefly reviewed.

MRI is a tool utilized with increasing frequency in clinical medicine and may be useful as a method of tracking exogenously delivered stromal cells to determine if they migrate preferentially to sites of inflammation. Various contrast agents can be used to enable MSC detection by MRI. These include positive contrast agents such as lanthanide chelates or manganese-containing compounds [207–209], negative contrast agents such as superparamagnetic iron oxide (SPIO) [210–213], ultra-small superparamagnetic iron oxide (USPIO) particles [213,214], or micrometer-sized iron oxide particles [215,216]. As is always the case when modifying cells, it is important to determine that MSCs retain their characteristic properties after cell labeling. Crabbe *et al.* labeled mouse embryonic stromal cells, rat multipotent adult progenitor cells, and mouse MSCs with different USPIO particles. With murine MSCs there was no difference in population doubling time at 7 days, nor was osteogenesis or adipogenesis affected [217]. In addition, the labeling of MSCs with SPIO was shown not to affect changes in surface marker expression or differentiation *in vitro* [218].

MRI has been used to study the behavior of neural progenitor cells *in vivo*, tracking migration of these cells to the olfactory bulb [216]. Similarly, MRI has been successfully used to track MSCs *in vivo*. The detection of magnetically labeled rat MSCs has been demonstrated in stroke models [219]. MSCs labeled with SPIO were infused into the ipsilateral internal carotid artery (ICA) of rats with an MCA occlusion. MRI was used before and 2–24 h after infusion and engraftment of labeled cells within the ICA vascular territory was detected by MRI and confirmed by high-resolution *ex vivo* MRI. Cells engrafted in the first pass

without entering the systemic circulation [219]. In another study, AMSCs delivered locally to a canine keratoconjunctivitis sicca (dry eye) model could be tracked locally with MRI initially. After 2 weeks the MRI signal was identified in the thymus and gastrointestinal tract [220].

Despite this, the role for MRI in this field remains uncertain. In the MCA stroke model, cerebral engraftment of MSCs was undetectable using MRI after intravenous administration [219]. Similarly, in a canine acute myocardial infarction (AMI) model in which ferumoxide-labeled canine MSCs were delivered intravenously they could not be subsequently detected by MRI, either *in vivo* or at postmortem [170]. Another study looked at the detection of SPIO-labeled stromal cells in a photothrombotic stroke model [210]. This model consists of an intravenous injection of a light-sensitive dye followed by focal illumination of the brain. The resulting dye activation causes oxidative damage, endothelial cell injury, and microvascular thrombosis. MRI detected hypointense lesions that correlated histologically with the formation of microglial scars. After initiation of an inflammatory cascade in the central nervous system the resident microglial cells become activated macrophages and infiltrate the injured tissue. These cells can incorporate ferric iron from the environment around the ischemic lesion. In this study, despite MRI lesions being identified, histological analysis was necessary to distinguish between labeled transplanted cells and immune cells in migroglial scars. Thus, caution is required when interpreting the *in vivo* monitoring of MSCs in models in which a strong inflammatory response is present [210]. Because iron oxide particles are retained in a tissue even if the grafted cell has died, false-positive signals remain a confounding factor [221]. In a nude rat model of antibody-induced arthritis, viable and nonviable human MSCs injected directly into the joint could not be distinguished with MRI [222].

SPECT and PET can also be used to determine the migration of MSCs to sites of inflammation. Previous studies in AMI models have demonstrated the potential usefulness of SPECT. In one study MSCs were labeled with $^{111}$In oxine. After confirmation that this did not alter their proliferation or differentiation *in vitro*, cells were delivered intravenously in a canine LAD occlusion model of AMI [170]. While MRI could not detect MSC migration in this model, SPECT was successfully used to demonstrate early uptake in the lungs and subsequent migration to the liver associated with migration to the infarcted heart [170]. SPECT has also been used to demonstrate human MSC homing to tumor sites after intraperitoneal delivery of $^{111}$In-oxine-labeled cells to mice with human xenografts of neuroblastoma [223].

PET imaging requires either activation of a tracer dye [224] or labeling with a radioactive tracer such as $^{18}$F-fludeoxyglucose [225]. While emissions from tracer dyes can be toxic and inhibit cellular function, labeling of murine MSCs with $^{18}$F-fludeoxyglucose does not affect cell viability, proliferation, or differentiation [221,225]. PET has been used to track MSC migration in models of malignancy and Parkinson's disease [226,227].

Finally, BLI may be used. This method of cell tracking requires the expression of a bioluminescence protein such as those from the luciferase family [221]. BLI has been shown to be useful in studying migration and engraftment of MSCs in neural tissue [228,229]; see Figures 22.4 and 22.5.

## 22.6 Optimizing homing

While work is ongoing to fully understand the mechanisms behind MSC homing to sites of inflammation, optimization of MSC homing is being developed (Figure 22.6). These include attempts to modulate the physiological barrier of blood vessels and the lung, manipulation of the inflammatory microenvironment of the body, and the augmentation of chemotactic signals from the site of inflammation [230].

### 22.6.1 Culture conditions

An initial approach augmenting MSC homing and engraftment is optimization of the culture conditions of MSCs. Two important factors in this regard include the passage and plating density of MSCs prior to systemic delivery. As previously discussed, freshly isolated MSCs have superior migratory potential that decreases rapidly as they are culture-expanded [181]. Low passage number and low-density MSC cultures express podocalyxin-like protein, 6-integrin (CD49f), 4-integrin (CD49d), c-Met, CXCR, and CX3CR1. They were previously shown to be involved in cell trafficking or tumor progression. These abilities are reduced as MSCs approach 100% confluence and with subsequent passages [231]. Increased confluence in culture

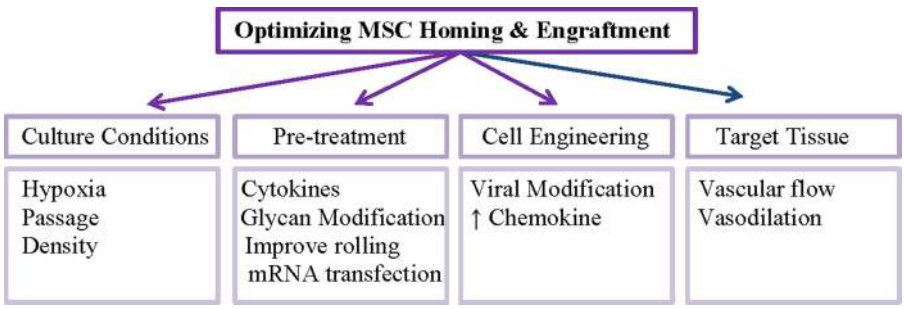



**Figure 22.6** Strategies for enhancing the homing of MSCs to sites of inflammation.

results in an upregulation of TIMP3, a natural inhibitor of MMP. The result of this is decreased transendothelial migration of MSCs *in vitro* – a process that is partly regulated by MMP-2 [85].

The downregulation of CXCR4 on cultured MSCs has been hypothesied as the mechanism the transformation of cultured MSCs into more differentiated cells [22]. However, it has been shown that culturing human MSCs as three-dimensional aggregates restores CXCR4 expression [232]. This effect was reversed when cells were re-plated in a monolayer, suggesting that cell functions are reversible and may adapt to cell culture conditions [232]; and see Chapters 7 and 8.

A novel method is the co-culture of MSCs with Sertoli cells. Sertoli cells support the development of sperm cells *in vivo* and have been shown to improve proliferation and survival of endothelial and neural cells *in vitro*. The co-culture of human UMSCs with Sertoli cells was associated with upregulation of *CXCR4*, *MMP2*, phospho-*p44MAPK*, and phospho-*p38MAPK* as well as other genes involved in cell proliferation and migration. There was also a small increase in transwell migration *in vitro* [233].

Finally, the role of short-term hypoxia in the culture of MSCs has been investigated. Hypoxic preconditioning of cardiac progenitor cells increased CXCR4 expression and augmented migration to infarcted myocardium *in vivo* [115]. Additionally, short term exposure of human MSCs to 1% oxygen was shown to result in an increased expression of CX3CR1 and CXCR4 at both mRNA and protein levels. After 24 h of hypoxia, *in vitro* migration to fractalkine and SDF-1 was increased [226].

While the ability to improve migration to inflamed tissues may be augmented by some of these modifications in culture conditions, the reality is that the low abundance of MSCs in bone marrow isolates and the likelihood that culture-expanded MSCs will be used in clinical settings represent a substantial challenge [232]. Therefore, work has focused on the engineering or pretreatment of MSCs, as well as on overcoming physiological host barriers.

### 22.6.2 Pretreatment of mesenchymal stromal cells

Pretreating MSCs during incubation has had promising and mixed results. Several studies have demonstrated an increase in MSC migration, both *in vitro* and *in vivo*, after preincubation with cytokines. A variety of MSCs have been used, including human adult BMSCs, human fetal BMSCs, human AMSCs, UCB MSCs and murine MSCs [80,119,122–124,234]. In studies with human BMSCs, cells were found to migrate in increased numbers toward chemokines *in vitro* after preincubation with TNF-α [80]. Naive MSCs migrated preferentially to growth factors PDGF-AB and insulin-like growth factor 1 (IGF-1), compared with their migration to chemokines RANTES, MDC, and SDF-1. An increase in migration towards growth factors was not seen after preincubation. An increase in migration toward chemokines was associated with an increase in MSC expression of CCR2, CCR3, and CCR4 as well as the membrane binding of RANTES. However, it was not associated with an increase in CXCR4 expression, leading the authors to suggest that TNF-α might modify the sensitivity to SDF-1 or modulate CXCR4 signal transduction [80]. However, in other studies, the expression of CXCR4 has been modified by pretreatment strategies [122,123,234]. In the first of these, both intracellular and surface expression of CXCR4 was increased after exposure of fetal BMSCs to a cytokine of cocktails in culture, which included Flt-3 ligand, SCF, IL-6, HGF, and IL-3, and resulted in an associated increase in migration toward SDF-1 *in vitro* [234]. An increase in CXCR4 mRNA and protein expression was demonstrated in UMSCs after preincubation with IL-1β [122]. This was associated with enhanced migration to an area of inflamed colon *in vivo* that could be abrogated by IL-1R1 siRNA [122]. Another strategy shown to improve MSC homing via upregulation of CXCR4 is the pretreatment of murine MSCs with IGF-1 resulting in a twofold increase in CXCR4 expression [123]. In this work, GDNF and TNF-α were also studied. While these did not increase CXCR4 expression, all three factors affected changes in the cytoskeleton of the MSCs with a more parallel orientation of filaments possibly indicative of an architectural remodeling necessary for the mechanical needs of migration [123]. Additionally, while only IGF-1 increased CXCR4 expression, pretreatment with any one of the three factors amplified the migration response to cisplatin-injured proximal tubular epithelial cells *in vitro* [123].

### 22.6.3 Cell engineering

As knowledge of factors involved in cell homing to injured tissue increases, so do attempts to modify MSCs to augment this migration process. Early studies looked at the ability of MSCs to migrate to bone marrow, including the use of α4 integrin and CD44 [235,236]. In the first of these studies murine MSCs were transduced with a recombinant adeno-associated virus (AAV2) expressing α4 integrin, which is known to play an important role in cell migration and invasion. *In vivo* studies in immunocompetent mice confirmed that, after intravenous delivery, significant enhancement of bone homing was seen after transduction. While this was not a disease or inflammation model, it confirmed the potential of this type of strategy to modify MSC behavior [235]. Subsequently, another group focused on CD44. This is expressed on MSCs but not the sialofucosylated glycoform of CD44 known as hematopoietic cell E-selectin/L-selectin ligand (HCELL), which is an E-selectin ligand expressed on hematopoietic cells. The HCELL glycoform was originally discovered on human hematopoietic stem cells and leukemic blast cells and was subsequently identified on cancer cells. HCELL functions as a bone marrow homing receptor, directing migration of human hematopoietic stem cells and mesenchymal stem/stromal cells to bone marrow. The authors converted the CD44 glycoform on MSCs into HCELL using a 1,3-fucosyltransferase preparation and specialized enzymatic conditions. Real-time intravital microscopy using NOD/SCID mice showed infiltration of the marrow with these cells within hours of transfusion. Again,

this was not a disease model but is an example of a method in which bone marrow tropism can be achieved by chemical engineering of glycans [236].

Owing to the important role of the CXCR4/SDF-1 axis in MSC migration, this pathway has been targeted by several groups to improve MSC homing. In one study, male rat MSCs were transduced with an adenoviral vector expressing CXCR4 and delivered intramyocardially in an LAD coronary artery ligation AMI model. Enhanced migration of the transduced cells toward SDF-1 was confirmed *in vitro* in cell migration assays. It was subsequently shown using FISH that, after intramyocardial delivery, cells expressing CXCR4 migrated significantly more than untransduced MSCs to the peri-infarct and infarct areas. Immunohistochemistry was used to suggest that infused MSCs contributed to the formation of new myocytes. In addition, cardiac function was significantly improved in rats receiving MSCs overexpressing CXCR4 [61]. Other groups have focused on viral vectors with more long-term gene expression, such as lentiviral and retroviral vectors. In early studies in an AMI model, MSCs transduced with a retrovirus expressing CXCR4 were delivered intravenously to a rat occlusion/reperfusion model of AMI. In comparison with untransduced MSCs, they homed in greater numbers toward the infarcted myocardium [237]. A model of acute liver injury was carried out using human bone-marrow-derived UE7T-13 cells immortalized by a retrovirus carrying human telomerase reverse transcriptase. A lentiviral vector was used to transduce MSCs to express CXCR4. These cells were shown to have a superior *in vitro* migration capacity toward SDF-1 and were subsequently delivered intravenously 24 h after an acute live injury in a nude mouse. Modified MSCs migrated to the liver in larger numbers than nonmodified MSCs. This was associated with reduced mortality and improved liver function [238]. Subsequently, a rat model of acute lung injury was used in which rat MSCs transduced to express CXCR4 with a lentiviral vector were delivered intravenously 1 h after injury induction. Expression of SDF-1 is known to be high in alveoli during acute lung injury and pulmonary fibrosis. Modified cells migrated at a higher rate to the lung, as demonstrated by immunohistochemistry and which correlated with *in vitro* migration assays [239].

Alternative methods for promoting CXCR4 expression by MSCs have been explored. Human UMSCs have been engineered to overexpress CXCR4 using a cationic liposomal reagent. In chemoinvasion assays there was a significant increase in the number of cells migrating toward an SDF-1 gradient compared with nontransfected cells [240]. Separately, a lipid–polyethylene glycol was used to apply recombinant CXCR4 to the surface of culture-expanded MSCs. This provided an ultrathin coating on the cell surface without covalent bonds or electrostatic interaction. Using these methods it was reported that recombinant CXCR4 could be incorporated into the cell surface membrane in approximately 10 min. Binding of SDF-1 was confirmed, and an *in vitro* assay demonstrated that modified MSCs migrated toward SDF-1 in a concentration-dependent manner. The authors hypothesized that this could be used for early administration of allogeneic MSCs, as SDF-1 expression is at its highest 48 h after infarction and a rapid method of cell engineering is needed [241].

Others studies have looked at the role of adhesion molecules in promoting homing, and attempts have been made to augment rolling mechanisms. It was hypothesized that coating MSCs with anti-addressin antibodies would promote more efficient delivery of MSCs to sites of inflammation in a murine model of inflammatory bowel disease. *In vivo* BLI and fluorescent imaging were used to determine that MSCs coated with VCAM showed a modest increase in migration to mesenteric lymph nodes and the colon. However, it is worth noting that in this case amelioration of disease was seen after delivery of MSCs coated with mucosal addressin cell adhesion molecule and not VCAM, suggesting that increased homing alone is not sufficient to modify disease outcomes [242].

Imparting a cell rolling characteristic to MSCs has been investigated by Sarkar *et al.* [243]. In this study, adult MSCs were generated to express functional rolling machinery, specifically P-selectin glycoprotein ligand-1 (PSGL-1) and sialyl Lewis X (SLeX) using mRNA. PSGL-1 facilitates the posttranslational modification of SLeX, creating a functional ligand for P-selectin and E-selectin and resulting in cell tethering and rolling on inflamed vascular endothelium. *In vitro* studies indicated that transfected MSCs showed a strong cell rolling response under physiological shear stress. *In vivo* using real-time confocal microscopy, transfected MSCs were seen to roll on inflamed endothelium. Furthermore, when injected intra-orbitally these cells migrated toward lipopolysaccharide-induced ear inflammation 30% more than unmanipulated MSCs after 2 h. When MSCs were triple transfected with PSGL-1, SLeX, and IL-10 a superior anti-inflammatory effect was observed. In this study, PSGL-1/SLeX MSCs demonstrated a 48.5% increase in homing to normal bone marrow compared with unmanipulated MSCs and an 88.3% increase in homing to irradiated bone marrow. These data strongly suggested that equipping MSCs with the homing ligands PSGL-1/SLeX by mRNA transfection significantly improved their homing to the marrow, especially under inflammatory conditions.

Overall, significant strides have been made in the field of cell engineering that are likely to play a role in the future of MSC therapies for inflammatory and ischemic pathologies [244]. The practical application of these strategies in terms of timing, scale and safety will need to be carefully addressed before they can be translated for clinical applications.

### 22.6.4 The host environment

Modifications of the host tissue may also be used to augment the migration of exogenously delivered MSCs. This may be by the use of medications designed to improve flow or through the overexpression of chemokines in target tissue.

In one study it was determined that MCP-3 acted via CCR1 and CCR2 on MSCs and could enhance migration *in vitro* in a

concentration-dependent manner. To test this *in vivo*, a rat LAD ligation AMI model was used. The potential role of MCP-3 was assessed by transplanting MCP-3-expressing cardiac fibroblasts into the infarct border zone 1 month after LAD ligation and induction of the infarct. In initial studies of a single infusion, MSCs were shown to migrate to the infarct. This was followed by a trial of serial infusions in an attempt to overcome lung trapping and the short half-life of MSCs. After six infusions over 12 days there were significantly more MSCs engrafted in the myocardium of animals that received MCP-3-expressing cardiac fibroblasts compared with control cardiac fibroblasts. This enhanced migration was associated with improved cardiac function in MCP-3-expressing hearts thought to be due to recruitment of myofibroblasts and remodeling of the collagen matrix. This suggests possible benefits from reestablishing MSC homing and engraftment remote from the acute injury [35]. Similar results were shown using a transgenic model in which MCP-1 was specifically expressed on myocardium. Adult BMSCs were delivered intravenously to these mice, and after 7 days engraftment of cells was visible only in the hearts of transgenic mice but not in the liver or spleen nor in control hearts [81].

The potential role of heparin and sodium nitroprusside has also been investigated. As blood vessels are the primary route of systemically delivered cells, the use of medications to ensure that vessels are free from debris and clot and to ensure adequate vasodilation to optimize passage of MSCs through capillary beds seems reasonable. As discussed earlier in this chapter, the trapping of MSCs in lung capillary beds after intravenous delivery is a significant barrier to mediating their effects in tissues other than the lungs. Using adipose cells capable of differentiation into mesenchymal and hematopoietic cells, the addition of heparin at the time of intravenously transplanted cells successfully aided homing in a model of acute liver injury and appeared to decrease lung trapping [198,245]. This was observed using *in vivo* fluorescence imaging.

Finally, in murine MSCs transfected with firefly luciferase, *in vivo* monitoring was used to assess cell distribution after intravenous injection. Cell trapping in the lungs was significantly reduced after pretreatment with sodium nitroprusside, potentially facilitating cell access to injured organs beyond the lungs [190].

## 22.7 Conclusions

MSCs are the ongoing focus of a major international effort to develop novel therapies for degenerative, inflammatory, and ischemic conditions. These cells hold the potential promise of regeneration, the modulation of damaging immune responses, and are readily isolated and cultured. There is ample evidence that the inflammatory environment augments migration both *in vitro* and *in vivo*. Whether present in endogenous reservoirs or introduced from exogenous or distant sources, MSCs are consistently exposed to tissue signals and interact with numerous immune cells and mediators that can influence their migratory behavior. *In vitro* there are multiple elements of the inflammatory milieu are at play, and they have important downstream effects. For example, the proinflammatory cytokines TNF-α and IL-1β have clear effects on MSC migration. Other effects, including the activation of the complement cascade and macrophage recruitment, appear to optimize the homing ability of MSCs.

There has been extensive *in vitro* work aimed at identifying the important factors that influence migration. This has resulted in a large number of potentially useful chemokine receptors on MSCs being detected. While these are often present in only small numbers, they appear to exert a significant effect on MSC behavior when stimulated by the appropriate ligand. Many of these ligands are endogenously upregulated in both ischemic and inflammatory tissues to create a positive cascade effect. A particular focus has been the interaction between SDF-1 and CXCR4. This has been shown to have a potent effect on cell behavior and has been adopted in cell engineering strategies designed to improve MSC homing. While an important pathway, it is likely that this effect works synergistically not only with other cytokine pathways, but also with the activation of TLRs, integrin receptors, and MMPs.

There has been a proliferation of *in vivo* MSC migration studies. Numerous models of inflammation have been studied, including cerebral and myocardial ischemia, AKI, and acute liver injury, as well as inflammatory arthritis. In almost all reported studies, *ex vivo* imaging techniques have demonstrated migration of cells to injured tissue. What has become apparent is the difficulty of determining the exact numbers of cells that do in fact migrate and indeed if the tracked cells are viable when they reach their target location. After intravenous injection the majority of MSCs are trapped in the capillary bed of the lung for up to 3 days and the number of MSCs reaching the target site and having a therapeutic effect is hard to gauge.

There are large variations between studies in terms of cell source, passage number, incubation protocols, the use of immunocompromised hosts, route and timing of delivery, and cell numbers infused. These may all act as confounding factors in the analysis of results. Real-time live imaging modalities will further improve our understanding of how these cells behave once administered to a new recipient. While MRI seems promising there have been some mixed results, as outlined earlier. In addition, the incorporation of ferric iron into macrophages and the retention of iron oxide particles in the tissue even after cell death remain important confounders. SPECT, PET, and BLI will likely prove to be more useful methods of tracking cell behavior *in vivo*.

While MSCs certainly have the potential and ability to migrate to sites of inflammation, the current challenge is the augmentation of this process by manipulation of either the MSC itself, the host environments through which they pass, or the tissues they are aiming to reach. While using very early passage MSCs is likely to optimize homing, it raises technical challenges around cell numbers and negates the benefits of using culture-expanded

MSCs. Modification of culture conditions is likely to prove useful and in particular the benefits of short-term hypoxia or cytokine pre-stimulation, as discussed earlier. The engineering of MSCs (see Chapters 23, 59, 60, and 61) to attenuate expression of chemokine receptors has been successful in improving migration, and while the laboratory methods of viral transduction are highly efficient, they will pose significant manufacturing and regulatory challenges for their translation to the clinic.

One of the most important issues to be addressed is that of accurate tracking of live cells in real time *in vivo* (see Chapter 24) and determination of the proximity to the target tissue needed for MSCs to exert their desired paracrine influence on host progenitor cells.

## References


1 F.P. Barry, J.M. Murphy. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* 2004;**36**:568–584.

2 F. Tögel, K. Weiss, Y. Yang, *et al.* Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol* 2007;**292**: F1626–F1635.

3 A.M. Hocking, N.S. Gibran. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp Cell Res*. 2010;**316**:2213–2219.

4 S.A. Wexler, C. Donaldson, P. Denning-Kendall, *et al.* Adult bone marrow is a rich source of human mesenchymal "stem" cells but umbilical cord and mobilized adult blood are not. *Br J Haematol* 2003;**121**:368–374.

5 L. da Silva Meirelles, P.C. Chagastelles, N.B. Nardi. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*. 2006;**119**:2204–2213.

6 M.J. Hoogduijn, M.J. Crop, A.M. Peeters, *et al.* Donor-derived mesenchymal stem cells remain present and functional in the transplanted human heart. *Am J Transplant* 2009;**9**:222–223.

7 J.J. Alm, H.M. Koivu, T.J. Heino, *et al.* Circulating plastic adherent mesenchymal stem cells in aged hip fracture patients. *J Orthop Res* 2010;**28**:1634–1642.

8 E. Eggenhofer, F. Luk, M.H. Dahlke, M.J. Hoogduijn. The life and fate of mesenchymal stem cells. *Front Immunol* 2014;**5**:148.

9 G.Y. Rochefort, B. Delorme, A. Lopez, *et al.* Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. *Stem Cells* 2006;**24**:2202–2208.

10 Y. Chen, L.X. Xiang, J.Z. Shao, *et al.* Recruitment of endogenous bone marrow mesenchymal stem cells towards injured liver. *J Cell Mol Med* 2010;**14**:1494–1508.

11 M. Gil-Ortega, L. Garidou, C. Barreau, *et al.* Native adipose stromal cells egress from adipose tissue in vivo: evidence during lymph node activation. *Stem Cells* 2013;**31**:1309–1320.

12 A. Augello, R. Tasso, S.M. Negrini, *et al.* Cell therapy using allogeneic bone marrow mesenchymal stem cells prevents tissue damage in collagen-induced arthritis. *Arthritis Rheum* 2007;**56**: 1175–1186.

13 E.M. Horwitz, D.J. Prockop, L.A. Fitzpatrick, *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfect. *Nat Med* 1999;**5**:309–313.

14 R.H. Lee, M.J. Seo, R.L. Reger, *et al.* Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proc Natl Acad Sci U S A* 2006;**103**:17438–17443.

15 R.S. Ripa, M. Haack-Sorensen, Y. Wang, *et al.* Bone marrow derived mesenchymal cell mobilization by granulocytecolony stimulating factor after acute myocardial infarction: results from the Stem Cells in Myocardial Infarction (STEMMI) trial. *Circulation* 2007;**116**: I24–I30.

16 M. Duijvestein, A.C. Vos, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010; **59**:1662–1669.

17 Y. Ikegame, K. Yamashita, S. Nakashima, *et al.* Fate of graft cells: what should be clarified for development of mesenchymal stem cell therapy for ischemic stroke? *Front Cell Neurosci* 2014;**8**:322.

18 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

19 D.J. Campbell, C.H. Kim, E.C. Butcher. Chemokines in the systemic organization of immunity. *Immunol Rev* 2003;**195**:58–71.

20 H.E. Broxmeyer, S. Cooper, D.M. Hass, *et al.* Experimental basis of cord blood transplantation. *Bone Marrow Transplant*. 2009;**44**: 627–633.

21 Z.C. Han, M. Lu, J. Li, *et al.* Platelet factor 4 and other CXC chemokines support the survival of normal hematopoietic cells and reduce the chemosensitivity of cells to cytotoxic agents. *Blood* 1997;**89**:2328–2335.

22 M. Honczarenko, Y. Le, M. Swierkowski, *et al.* Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells* 2006;**24**:1030–1041.

23 T.P. Garrington, G.L. Johnson. Organization and regulation of mitogen-activated protein kinase signaling pathways. *Curr Opin Cell Biol* 1999;**11**:211–218.

24 A.E. Aplin, A. Howe, S.K. Alahari, RL Juliano. Signal transduction and signal modulation by cell adhesion receptors: the role of integrins, cadherins, immunoglobulin-cell adhesion molecules, and selectins. *Pharmacol Rev* 1998;**50**:197–263.

25 W.J. Leonard, J.J. O'Shea. Jaks and STATs: biological implications. *Annu Rev Immunol* 1998;**16**:293–322.

26 I. Von Lüttichau, M. Notohamiprodjo, A. Wechselberger, *et al.* Human adult $CD34^-$ progenitor cells functionally express the chemokine receptors CCR1, CCR4, CCR7, CXCR5, and CCR10 but not CXCR4. *Stem Cells Dev* 2005;**14**:329–336.

27 F. Xu, J. Shi, B. Yu, *et al.* Chemokines mediate mesenchymal stem cell migration toward gliomas in vitro. *Oncol Rep* 2010;**23**: 1561–1567.

28 F. Zhang, S. Tsai, K. Kato, *et al.* Transforming growth factor-β promotes recruitment of bone marrow cells and bone marrow-derived mesenchymal stem cells through stimulation of MCP-1 production in vascular smooth muscle cells. *J Biol Chem* 2009;**84**: 17564–17574.

29 R.M. Dwyer, S.M. Potter-Beirne, K.A. Harrington, *et al.* Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clin Cancer Res* 2007;**13**:5020–5027.

30 J.F. Ji, B.P. He, S.T. Dheen, S.S. Tay. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* 2004;**22**:415–427.

31 A. Mor, S.B. Abramson, M.H. Pillinger. The fibroblast-like synovial cell in rheumatoid arthritis: a key player in inflammation and joint destruction. *Clin Immunol* 2005;**115**:118–128.

32 J. Ringe, S. Strassburg, K. Neumann, *et al.* Towards *in situ* tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 2007;**101**:135–146.

33 S.L. Deshmane, S. Kremlev, S. Amini, B.E. Sawaya. Monocyte chemoattractant protein-1 (MCP-1): an overview. *J Interferon Cytokine Res* 2009;**29**:313–326.

34 T. Akahoshi, C. Wada, H. Endo, *et al.* Expression of monocyte chemotactic and activating factor in rheumatoid arthritis. *Regulation of its production in synovial cells by interleukin-1 and tumor necrosis factor. Arthritis Rheum* 1993;**36**:762–771.

35 S. Schenk, N. Mal, A. Finan, *et al.* Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* 2007;**25**:245–251.

36 M. Maurer, E. von Stebut. Macrophage inflammatory protein-1. *Int J Biochem Cell Biol* 2004;**36**:1882–1886.

37 A.E. Koch, S.L. Kunkel, L.A. Harlow, *et al.* Macrophage inflammatory protein-1α. A novel chemotactic cytokine for macrophages in rheumatoid arthritis. *J Clin Invest* 1994;**93**:921–928.

38 V. Sordi, M.L. Malosio, F. Marchesi, *et al.* Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005;**106**:419–427.

39 R.A. Boomsma, D.L. Geenen. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. *PLoS ONE* 2012;**7**: e35685.

40 T. Takano, Y.J. Li, A. Kukita, *et al.* Mesenchymal stem cells markedly suppress inflammatory bone destruction in rats with adjuvant-induced arthritis. *Lab Invest* 2014;**94**:286–296.

41 C.C. Bleul, R.C. Fuhlbrigge, J.M. Casasnovas, A. *et al.* A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). *J Exp Med* 1996;**184**:1101–1109.

42 E. Oberlin, A. Amara, F. Bachelerie, *et al.* The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. *Nature* 1996;**382**:833–835.

43 A. Dar, P. Goichberg, V. Shinder, *et al.* Chemokine receptor CXCR4-dependent internalization and resecretion of functional chemokine SDF-1 by bone marrow endothelial and stromal cells. *Nat Immunol* 2005;**6**:1038–1046.

44 K. Imai, M. Kobayashi, J. Wang, *et al.* Selective secretion of chemoattractants for haemopoietic progenitor cells by bone marrow endothelial cells: a possible role in homing of haemopoietic progenitor cells to bone marrow. *Br J Haematol* 1999;**106**: 905–9–11.

45 A. Peled, V. Grabovsky, L. Habler, *et al.* The chemokine SDF-1 stimulates integrin-mediated arrest of $CD34^+$ cells on vascular endothelium under shear flow. *J Clin Invest* 1999;**104**:1199–1211.

46 T. Ponomaryov, A. Peled, I. Petit, *et al.* Induction of the chemokine stromal-derived factor-1 following DNA damage improves human stem cell function. *J Clin Invest* 2000;**106**:1331–1339.

47 I. Petit, M. Szyper-Kravitz, A. Nagler, *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 2002;**3**:687–694.

48 O. Salvucci, L. Yao, S. Villalba, *et al.* Regulation of endothelial cell branching morphogenesis by endogenous chemokine stromal-derived factor-1. *Blood* 2002;**99**:2703–2711.

49 J. Hesselgesser, M. Halks-Miller, V. DelVecchio, *et al.* CD4-independent association between HIV-1 gp120 and CXCR4: functional chemokine receptors are expressed in human neurons. *Curr Biol* 1997;**7**:112–121.

50 M. Kucia, J. Ratajczak, R. Reca, *et al.* Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF 1 gradient and are mobilized into peripheral blood during stress and tissue injury. *Blood Cells Mol Dis* 2004;**32**:52–57.

51 J. Cashman, I. Clark-Lewis, A. Eaves, C. Eaves. Stromal-derived factor 1 inhibits the cycling of very primitive human hematopoietic cells in vitro and in NOD/SCID mice. *Blood* 2002;**99**:792–799.

52 J. Ma, J. Ge, S. Zhang, *et al.* Time course of myocardial stromal cell-derived factor 1 expression and beneficial effects of intravenously administered bone marrow stem cells in rats with experimental myocardial infarction. *Basic Res Cardiol* 2005;**100**:217–223.

53 F. Tögel, J. Isaac, Z. Hu, *et al.* Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int* 2005;**67**:1772–1784.

54 L.H. Shen, Y. Li, J. Chen, *et al.* Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab* 2007;**27**:6–13.

55 T. Kitaori, H. Ito, E.M. Schwarz, *et al.* Stromal cell-derived factor 1/CXCR4 signaling is critical for the recruitment of mesenchymal stem cells to the fracture site during skeletal repair in a mouse model. *Arthritis Rheum* 2009;**60**:813–823.

56 N. Liu, J. Tian, J. Cheng, J. Zhang. Migration of CXCR4 gene-modified bone marrow-derived mesenchymal stem cells to the acute injured kidney. *J Cell Biochem* 2013;**114**:2677–2689.

57 R.F. Wynn, C.A. Hart, C. Corradi-Perini, *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 2004;**104**:2643–2645.

58 C.H. Ryu, S.A. Park, S.M. Kim, *et al.* Migration of human umbilical cord blood mesenchymal stem cells mediated by stromal cell-derived factor-1/CXCR4 axis via Akt, ERK, and p38 signal transduction pathways. *Biochem Biophys Res Commun* 2010;**398**: 105–110.

59 A. Kortesidis, A. Zannettino, S. Isenmann, *et al.* Stromal-derived factor-1 promotes the growth, survival, and development of human bone marrow stromal stem cells. *Blood* 2005;**105**:3793–3801.

60 A. Jaerve, J. Schira, H.W. Müller. Concise review: the potential of stromal cell-derived factor 1 and its receptors to promote stem cell functions in spinal cord repair. *Stem Cells Transl Med* 2012;**1**:732–739.

61 D. Zhang, G.C. Fan, X. Zhou, *et al.* Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. *J Mol Cell Cardiol* 2008;**44**:281–292.

62 V.M. Tysseling, D. Mithal, V. Sahni, *et al.* SDF1 in the dorsal corticospinal tract promotes $CXCR4^+$ cell migration after spinal cord injury. *J Neuroinflammation* 2011;**8**:16.

63 F. Knerlich-Lukoschus, B. von der Ropp-Brenner, R. Lucius, *et al.* Chemokine expression in the white matter spinal cord precursor

niche after force-defined spinal cord contusion injuries in adult rats. *Glia* 2010;**58**:916–931.
64 R. Pal, C. Gopinath, N.M. Rao, *et al.* Functional recovery after transplantation of bone marrow-derived human mesenchymal stromal cells in a rat model of spinal cord injury. *Cytotherapy* 2010;**12**:792–806.
65 D. Cízková, J. Rosocha, I. Vanický, *et al.* Transplants of human mesenchymal stem cells improve functional recovery after spinal cord injury in the rat. *Cell Mol Neurobiol* 2006;**26**:1167–1180.
66 C.Y. Ho, A. Sanghani, J. Hua, *et al.* Mesenchymal stem cells with increased stromal cell-derived factor 1 expression enhanced fracture healing. *Tissue Eng Part A* 2015;**21**(3–4):594–602.
67 C. Madjdpour, U.R. Jewell, S. Kneller, *et al.* Decreased alveolar oxygen induces lung inflammation. *Am J Physiol Lung Cell Mol Physiol* 2003;**284**:L360–L367.
68 G.L. Semenza. HIF-1 and tumor progression: pathophysiology and therapeutics. *Trends Mol Med* 2002;**8**:S62–S67.
69 C.T. Ng, M. Biniecka, A. Kennedy, *et al.* Synovial tissue hypoxia and inflammation *in vivo*. *Ann Rheum Dis* 2010;**69**:1389–1395.
70 A. Sica, A. Saccani, A. Mantovani. Tumor-associated macrophages: a molecular perspective. *Int Immunopharmacol* 2002;**2**:1045–1054.
71 T. Schioppa, B. Uranchimeg, A. Saccani, *et al.* Regulation of the chemokine receptor CXCR4 by hypoxia. *J Exp Med* 2003;**98**: 1391–1402.
72 Y.S. Oh, H.Y. Kim, I.C. Song, *et al.* Hypoxia induces CXCR4 expression and biological activity in gastric cancer cells through activation of hypoxia-inducible factor-1α. *Oncol Rep* 2012;**28**: 2239–2246.
73 M.A. Krook, L.A. Nicholls, C.A. Scannell, *et al.* Stress-induced CXCR4 promotes migration and invasion of Ewing sarcoma. *Mol Cancer Res* 2014;**12**:953–964.
74 N. Wright, T.L. de Lera, C. García-Moruja, *et al.* Transforming growth factor-beta1 down-regulates expression of chemokine stromal cell-derived factor-1: functional consequences in cell migration and adhesion. *Blood*. 2003;**102**:1978–1984.
75 A.L. Ponte, E. Marais, N. Gallay, *et al.* The *in vitro* migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 2007;**25**:1737–1745.
76 I. Petit, P. Goichberg, A. Spiegel, *et al.* Atypical PKC-ζ regulates SDF-1-mediated migration and development of human $CD34^+$ progenitor cells. *J Clin Invest* 2005;**115**:168–176.
77 Y. Wu, R.C. Zhao. The role of chemokines in mesenchymal stem cell homing to myocardium. *Stem Cell Rev* 2012;**8**:243–250.
78 B.R. Son, L.A. Marquez-Curtis, M. Kucia, *et al.* Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells* 2006;**24**:1254–1264.
79 J. Stagg, J. Galipeau. Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. *Curr Mol Med* 2013;**13**:856–867.
80 H. Smith, C. Whittall, B. Weksler, J. Middleton. Chemokines stimulate bidirectional migration of human mesenchymal stem cells across bone marrow endothelial cells. *Stem Cells Dev* 2012;**21**: 476–486.
81 F. Belema-Bedada, S. Uchida, A. Martire, *et al.* Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell* 2008;**2**:566–575.
82 J.E. Ip, Y. Wu, J. Huang, *et al.* Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell* 2007;**18**:2873–2882.
83 S.L. Tomchuck, K.J. Zwezdaryk, S.B. Coffelt, *et al.* Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells* 2008;**26**:99–107.
84 B. Annabi, Y.T. Lee, S. Turcotte, *et al.* Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. *Stem Cells* 2003;**21**:337–347.
85 A. De Becker, P. Van Hummelen, M. Bakkus, *et al.* Migration of culture-expanded human mesenchymal stem cells through bone marrow endothelium is regulated by matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-3. *Haematologica* 2007;**92**:440–449.
86 C. Ries, V. Egea, M. Karow, *et al.* MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells: differential regulation by inflammatory cytokines. *Blood* 2007;**109**:4055–4063.
87 S.J. Park, K.J. Kim, W.U. Kim, C.S. Cho. Interaction of mesenchymal stem cells with fibroblast-like synoviocytes via cadherin-11 promotes angiogenesis by enhanced secretion of placental growth factor. *J Immunol* 2014;**192**:3003–3010.
88 P. Cipriani, A. Marrelli, P.D. Benedetto, *et al.* Scleroderma mesenchymal stem cells display a different phenotype from healthy controls; implications for regenerative medicine. *Angiogenesis* 2013;**16**:595–607.
89 E. Lombardo, O. DelaRosa, P. Mancheño-Corvo, *et al.* Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. *Tissue Eng Part A* 2009;**15**:1579–1589.
90 A. Augello, R. Tasso, S.M. Negrini, *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;**35**:1482–1490.
91 M.A. González, E. Gonzalez-Rey, L. Rico, *et al.* Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009;**136**:978–989.
92 C.A. Opitz, U.M. Litzenburger, C. Lutz, *et al.* Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-β and protein kinase R. *Stem Cells* 2009;**27**:909–919.
93 I.F. Mo, K.H. Yip, W.K. Chan, *et al.* Prolonged exposure to bacterial toxins downregulated expression of toll-like receptors in mesenchymal stromal cell-derived osteoprogenitors. *BMC Cell Biol* 2008;**9**:52.
94 H. Hwa Cho, Y.C. Bae, J.S. Jung. Role of toll-like receptors on human adipose-derived stromal cells. *Stem Cells* 2006;**24**:2744–2752.
95 M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, *et al.* Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 2007;**109**:1422–1432.
96 H. Nagase, J.F. Woessner. Matrix metalloproteinases. *J Biol Chem* 1999;**274**:21491–21494.
97 C. Faveeuw, G. Preece, A. Ager. Transendothelial migration of lymphocytes across high endothelial venules into lymph nodes is affected by metalloproteinases. *Blood* 2001;**98**:688–695.
98 D. Leppert, E. Waubant, R. Galardy, *et al.* T cell gelatinases mediate basement membrane transmigration *in vitro*. *J Immunol* 1995;**154**: 4379–4389.

99 A. Janowska-Wieczorek, L.A. Marquez, A. Dobrowsky, *et al.* Differential MMP and TIMP production by human marrow and peripheral blood $CD34^+$ cells in response to chemokines. *Exp Hematol* 2000;**28**:1274–1285.

100 A. Janowska-Wieczorek, A. Matsuzaki, L.A. Marquez. The hematopoietic microenvironment: matrix metalloproteinases in the hematopoietic microenvironment. *Hematology* 2000;**4**:515–527.

101 D. Maglione, V. Guerriero, G. Viglietto, *et al.* Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor. *Proc Natl Acad Sci U S A* 1991;**88**:9267–9271.

102 J. DiSalvo, M.L. Bayne, G. Conn, *et al.* Purification and characterization of a naturally occurring vascular endothelial growth factor·-placenta growth factor heterodimer. *J Biol Chem* 1995;**270**: 7717–7723.

103 S.K. Selvaraj, R.K. Giri, N. Perelman, *et al.* Mechanism of monocyte activation and expression of proinflammatory cytochemokines by placenta growth factor. *Blood* 2003;**102**:1515–1524.

104 S.A. Yoo, H.J. Yoon, H.S. Kim, *et al.* Role of placenta growth factor and its receptor flt-1 in rheumatoid inflammation: a link between angiogenesis and inflammation. *Arthritis Rheum* 2009;**60**:345–354.

105 N. Hata, N. Shinojima, J. Gumin, *et al.* Platelet-derived growth factor BB mediates the tropism of human mesenchymal stem cells for malignant gliomas. *Neurosurgery* 2010;**66**:144–156.

106 J.M. Lee, B.S. Kim, H. Lee, G.I. Im. *In vivo* tracking of mesenchymal stem cells using fluorescent nanoparticles in an osteochondral repair model. *Mol Ther* 2012;**20**:1434–1442.

107 W.G. Zhang, L. He, X.M. Shi, *et al.* Regulation of transplanted mesenchymal stem cells by the lung progenitor niche in rats with chronic obstructive pulmonary disease. *Respir Res* 2014;**15**:33.

108 M. Kanichai, D. Ferguson, P.J. Prendergast, V.A. Campbell. Hypoxia promotes chondrogenesis in rat mesenchymal stem cells: a role for AKT and hypoxia-inducible factor (HIF)-1α. *J Cell Physiol* 2008;**216**:708–715.

109 L.B. Buravkova, E.R. Andreeva, V. Gogvadze, B. Zhivotovsky. Mesenchymal stem cells and hypoxia: where are we? *Mitochondrion* 2014;**19**:105–112.

110 S.H. Lee, Y.J. Lee, C.H. Song, *et al.* Role of FAK phosphorylation in hypoxia-induced hMSCS migration: involvement of VEGF as well as MAPKS and eNOS pathways. *Am J Physiol Cell Physiol* 2010;**298**: C847–C856.

111 K.M. Peterson, A. Aly, A. Lerman, *et al.* Improved survival of mesenchymal stromal cell after hypoxia preconditioning: role of oxidative stress. *Life Sci* 2011;**88**:65–73.

112 C. Busletta, E. Novo, L. Valfrè Di Bonzo, *et al.* Dissection of the biphasic nature of hypoxia-induced motogenic action in bone marrow-derived human mesenchymal stem cells. *Stem Cells* 2011;**29**:952–963.

113 W. Zhang, X. Su, Y. Gao, *et al.* Berberine protects mesenchymal stem cells against hypoxia-induced apoptosis *in vitro*. *Biol Pharm Bull* 2009;**32**:1335–1342.

114 W. Chang, B.W. Song, S. Lim, *et al.* Mesenchymal stem cells pretreated with delivered Hph-1-Hsp70 protein are protected from hypoxia-mediated cell death and rescue heart functions from myocardial injury. *Stem Cells* 2009;**27**:2283–2292.

115 L. Wang, Y. Li, J. Chen, *et al.* Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. *Exp Hematol* 2002;**30**:831–836.

116 S.C. Hung, R.R. Pochampally, S.C. Hsu, *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* 2007;**2**:e416.

117 Y.L. Tang, W. Zhu, M. Cheng, *et al.* Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. *Circ Res* 2009;**104**:1209–1216.

118 W. Böcker, D. Docheva, W.C. Prall, *et al.* IKK-2 is required for TNF-α-induced invasion and proliferation of human mesenchymal stem cells. *J Mol Med (Berl)* 2008;**86**:1183–1192.

119 S.J. Baek, S.K. Kang, J.C. Ra. *In vitro* migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors. *Exp Mol Med* 2011;**43**:596–603.

120 Q. Xiao, S.K. Wang, H. Tian, *et al.* TNF-α increases bone marrow mesenchymal stem cell migration to ischemic tissues. *Cell Biochem Biophys* 2012;**62**:409–414.

121 R. Carrero, I. Cerrada, E. Lledó, *et al.* IL1β induces mesenchymal stem cells migration and leucocyte chemotaxis through NF-κB. *Stem Cell Rev* 2012;**8**:905–916.

122 H. Fan, G. Zhao, L. Liu, *et al.* Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis. *Cell Mol Immunol* 2012;**9**:473–481.

123 C. Xinaris, M. Morigi, V. Benedetti, *et al.* A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. *Cell Transplant* 2013;**22**:423–436.

124 C.B. Sullivan, R.M. Porter, C.H. Evans, *et al.* TNFα and IL-1β influence the differentiation and migration of murine MSCs independently of the NF-κB pathway. *Stem Cell Res Ther* 2014;**5**:104.

125 E. Jones, S.M. Churchman, A. English, *et al.* Mesenchymal stem cells in rheumatoid synovium: enumeration and functional assessment in relation to synovial inflammation level. *Ann Rheum Dis* 2010;**69**:450–457.

126 L. Zhao, J. Huang, H. Zhang, *et al.* Tumor necrosis factor inhibits mesenchymal stem cell differentiation into osteoblasts via the ubiquitin E3 ligase Wwp1. *Stem Cells* 2011;**29**:1601–1610.

127 D.C. Lacey, P.J. Simmons, S.E. Graves, J.A. Hamilton. Proinflammatory cytokines inhibit osteogenic differentiation from stem cells: implications for bone repair during inflammation. *Osteoarthritis Cartilage* 2009;**17**:735–742.

128 F. Djouad, V. Fritz, F. Apparailly, *et al.* Reversal of the immunosuppressive properties of mesenchymal stem cells by tumor necrosis factor α in collagen-induced arthritis. *Arthritis Rheum* 2005;**52**:1595–1603.

129 Y. Liu, Z.P. Han, S.S. Zhang, *et al.* Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem* 2011;**286**:25007–25015.

130 H. Lim, H.P. Kim. Matrix metalloproteinase-13 expression in IL-1β-treated chondrocytes by activation of the p38 MAPK/c-Fos/AP-1 and JAK/STAT pathways. *Arch Pharm Res* 2011;**34**:109–117.

131 E.S. Welf, S. Ahmed, H.E. Johnson, *et al.* Migrating fibroblasts reorient directionality by a metastable, PI3K-dependent mechanism. *J Cell Biol* 2012;**197**:105–114.

132 G.S. Firestein. NF-κB: Holy Grail for rheumatoid arthritis? *Arthritis Rheum* 2004;**50**:2381–2386.

133 Y. Li, Y.H. Liu, Z.J. Li, *et al. Staphylococcus aureus* infection of intestinal epithelial cells induces human umbilical cord-derived mesenchymal stem cell migration. *Int Immunopharmacol* 2013;**15**: 176–181.

134 P. Gasque. Complement: a unique innate immune sensor for danger signals. *Mol Immunol* 2004;**41**:1089–1098.

135 Z. Vegh, R.R. Kew, B.L. Gruber, B. Ghebrehiwet. Chemotaxis of human monocyte-derived dendritic cells to complement component C1q is mediated by the receptors gC1qR and cC1qR. *Mol Immunol* 2006;**43**:1402–1407.

136 S. Liu, J. Wu, T. Zhang, *et al.* Complement C1q chemoattracts human dendritic cells and enhances migration of mature dendritic cells to CCL19 via activation of AKT and MAPK pathways. *Mol Immunol* 2008;**46**:242–249.

137 B. Ghebrehiwet, R.R. Kew, B.L. Gruber, *et al.* Murine mast cells express two types of C1q receptors that are involved in the induction of chemotaxis and chemokinesis. *J Immunol* 1995;**155**: 2614–2619.

138 P. Kuna, M. Iyer, E.I. Peerschke, *et al.* Human C1q induces eosinophil migration. *Clin Immunol Immunopathol* 1996;**81**:48–54.

139 L.E. Leigh, B. Ghebrehiwet, T.P. Perera, *et al.* C1q-mediated chemotaxis by human neutrophils: involvement of gClqR and G-protein signalling mechanisms. *Biochem J* 1998;**330**:247–254.

140 I.U. Schraufstatter, R.G. Discipio, M. Zhao, S.K. Khaldoyanidi. C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. *J Immunol* 2009;**182**:3827–3836.

141 Y. Qiu, L.A. Marquez-Curtis, A. Janowska-Wieczorek. Mesenchymal stromal cells derived from umbilical cord blood migrate in response to complement C1q. *Cytotherapy* 2012;**14**:285–295.

142 M.A. Soland, M. Bego, E. Colletti, *et al.* Mesenchymal stem cells engineered to inhibit complement-mediated damage. *PLoS ONE* 2013;**8**:e60461.

143 Y. Li, F. Lin. Mesenchymal stem cells are injured by complement after their contact with serum. *Blood* 2012;**120**:3436–3443.

144 K. Anton, J. Glod. Targeting the tumor stroma in cancer therapy. *Curr Pharm Biotechnol* 2009;**10**:185–191.

145 K. Anton, D. Banerjee, J. Glod. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. *PLoS ONE* 2012;**7**:e35036.

146 E. Angst, H.A. Reber, O.J. Hines, G. Eibl. Mononuclear cell-derived interleukin-1β confers chemoresistance in pancreatic cancer cells by upregulation of cyclooxygenase-2. *Surgery* 2008;**144**:57–65.

147 R. Adamson. Role of macrophages in normal wound healing: an overview. *J Wound Care* 2009;**18**:349–351.

148 K. Ley, C. Laudanna, M.I. Cybulsky, S. Nourshargh. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;**7**:678–689.

149 C.V. Carman. Mechanisms for transcellular diapedesis: probing and pathfinding by "invadosome-like protrusions". *J Cell Sci* 2009;**122**:3025–3035.

150 B. Rüster, S. Göttig, R.J. Ludwig, *et al.* Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006;**108**:3938–3944.

151 V.F. Segers, I. Van Riet, L.J. Andries, *et al.* Mesenchymal stem cell adhesion to cardiac microvascular endothelium: activators and mechanisms. *Am J Physiol Heart Circ Physiol* 2006;**290**: H1370–H1377.

152 A. Schmidt, D. Ladage, C. Steingen, *et al.* Mesenchymal stem cells transmigrate over the endothelial barrier. *Eur J Cell Biol* 2006;**85**: 1179–1188.

153 C. Steingen, F. Brenig, L. Baumgartner, *et al.* Characterization of key mechanisms in transmigration and invasion of mesenchymal stem cells. *J Mol Cell Cardiol* 2008;**44**:1072–1084.

154 G.S. Teo, J.A. Ankrum, R. Martinelli, *et al.* Mesenchymal stem cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells* 2012;**30**:2472–2486.

155 G. Charras, E. Paluch. Blebs lead the way: how to migrate without lamellipodia. *Nat Rev Mol Cell Biol* 2008;**9**:730–736.

156 O.T. Fackler, R. Grosse. Cell motility through plasma membrane blebbing. *J Cell Biol* 2008;**181**:879–884.

157 E. Sahai, C.J. Marshall. Differing modes of tumour cell invasion have distinct requirements for Rho/ROCK signalling and extracellular proteolysis. *Nat Cell Biol* 2003;**5**:711–719.

158 S.M. Devine, C. Cobbs, M. Jennings, *et al.* Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003;**101**:2999–3001.

159 S. François, M. Bensidhoum, M. Mouiseddine, *et al.* Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* 2006;**24**:1020–1029.

160 M. Studeny, F.C. Marini, J.L. Dembinski, *et al.* Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* 2004;**96**: 1593–1603.

161 A. Nakamizo, F. Marini, T. Amano, *et al.* Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;**65**:3307–3318.

162 J. Bayo, E. Fiore, J.B. Aquino, *et al.* Human umbilical cord perivascular cells exhibited enhanced migration capacity towards hepatocellular carcinoma in comparison with bone marrow mesenchymal stromal cells: a role for autocrine motility factor receptor. *Biomed Res Int* 2014;**2014**:837420.

163 M.J. Lathrop, E.K. Sage, S.L. Macura, *et al.* Antitumor effects of TRAIL-expressing mesenchymal stromal cells in a mouse xenograft model of human mesothelioma. *Cancer Gene Ther* 2015;**22**:44–54.

164 Y. Zhu, M. Cheng, Z. Yang, *et al.* Mesenchymal stem cell-based NK4 gene therapy in nude mice bearing gastric cancer xenografts. *Drug Des Devel Ther* 2014;**8**:2449–2462.

165 Y. Omori, O. Honmou, K. Harada, *et al.* Optimization of a therapeutic protocol for intravenous injection of human mesenchymal stem cells after cerebral ischemia in adult rats. *Brain Res* 2008;**1236**:30–38.

166 V. Donega, C.T. van Velthoven, C.H. Nijboer, *et al.* Intranasal mesenchymal stem cell treatment for neonatal brain damage: long-term cognitive and sensorimotor improvement. *PLoS ONE* 2013;**8**: e51253.

167 I.M. Barbash, P. Chouraqui, J. Baron, *et al.* Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 2003;**108**:863–868.

168 N. Nagaya, T. Fujii, T. Iwase, *et al.* Intravenous administration of mesenchymal stem cells improves cardiac function in rats with acute myocardial infarction through angiogenesis and myogenesis. *Am J Physiol Heart Circ Physiol* 2004;**287**:H2670–H2676.

169 W. Jiang, A. Ma, T. Wang, *et al.* Intravenous transplantation of mesenchymal stem cells improves cardiac performance after acute myocardial ischemia in female rats. *Transpl Int* 2006;**19**:570–580.

170 D.L. Kraitchman, M. Tatsumi, W.D. Gilson, *et al.* Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* 2005;**112**:1451–1461.

171 E. Eggenhofer, V. Benseler, A. Kroemer, *et al.* Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* 2012;**3**:297.

172 M.B. Herrera, B. Bussolati, S. Bruno, *et al.* Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. *Kidney Int* 2007;**72**:430–441.

173 M. Morigi, M. Introna, B. Imberti, *et al.* Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice. *Stem Cells* 2008;**26**:2075–2082.

174 M. Morigi, B. Imberti, C. Zoja, *et al.* Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol* 2004;**15**:1794–1804.

175 C. Sullivan, F. Barry, T. Ritter, *et al.* Allogeneic murine mesenchymal stem cells: migration to inflamed joints *in vivo* and amelioration of collagen induced arthritis when transduced to express CTLA4Ig. *Stem Cells Dev* 2013;**22**:3203–3213.

176 G. Constantin, S. Marconi, B. Rossi, *et al.* Adipose-derived mesenchymal stem cells ameliorate chronic experimental autoimmune encephalomyelitis. *Stem Cells* 2009;**27**:2624–2635.

177 E. Zappia, S. Casazza, E. Pedemonte, *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 2005;**106**:1755–1761.

178 A. Mahmood, D. Lu, C. Qu, *et al.* Human marrow stromal cell treatment provides long-lasting benefit after traumatic brain injury in rats. *Neurosurgery* 2005;**57**:1026–1031.

179 C. De Bari, F. Dell'Accio, F. Vandenabeele, *et al.* Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* 2003;**160**:909–918.

180 L.A. Ortiz, F. Gambelli, C. McBride, *et al.* Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**:8407–8411.

181 W.J. Rombouts, R.E. Ploemacher. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* 2003;**17**:160–170.

182 Y. Horita, O. Honmou, K. Harada, *et al.* Intravenous administration of glial cell line-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in the adult rat. *J Neurosci Res* 2006;**84**:1495–1504.

183 M. Kanehira, H. Xin, K. Hoshino, *et al.* Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. *Cancer Gene Ther* 2007;**14**:894–903.

184 M. Sasaki, R. Abe, Y. Fujita, *et al.* Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol* 2008;**180**:2581–2587.

185 M.R. Loebinger, P.G. Kyrtatos, M. Turmaine, *et al.* Magnetic resonance imaging of mesenchymal stem cells homing to pulmonary metastases using biocompatible magnetic nanoparticles. *Cancer Res* 2009;**69**:8862–8867.

186 M. Maumus, J.A. Peyrafitte, R. D'Angelo, *et al.* Native human adipose stromal cells: localization, morphology and phenotype. *Int J Obes (Lond)* 2011;**35**:1141–1153.

187 J. Braun, A. Kurtz, N. Barutcu, *et al.* Concerted regulation of CD34 and CD105 accompanies mesenchymal stromal cell derivation from human adventitial stromal cell. *Stem Cells Dev* 2013;**22**: 815–827.

188 D.C. Colter, R. Class, C.M. DiGirolamo, D.J. Prockop. Rapid expansion of recycling stem cells in cultures of plastic-adherent cells from human bone marrow. *Proc Natl Acad Sci U S A* 2000;**97**:3213–3218.

189 M.J. Crop, C.C. Baan, S.S. Korevaar, *et al.* Inflammatory conditions affect gene expression and function of human adipose tissue-derived mesenchymal stem cells. *Clin Exp Immunol* 2010;**162**: 474–486.

190 S. Schrepfer, T. Deuse, H. Reichenspurner, *et al.* Stem cell transplantation: the lung barrier. *Transplant Proc* 2007;**39**:573–576.

191 L. de Girolamo, S. Lopa, E. Arrigoni, *et al.* Human adipose-derived stem cells isolated from young and elderly women: their differentiation potential and scaffold interaction during *in vitro* osteoblastic differentiation. *Cytotherapy* 2009;**11**:793–803.

192 F.Z. Asumda, P.B. Chase. Age-related changes in rat bone-marrow mesenchymal stem cell plasticity. *BMC Cell Biol* 2011;**12**:44.

193 M. Lavasani, A.R. Robinson, A. Lu, *et al.* Muscle-derived stem/progenitor cell dysfunction limits healthspan and lifespan in a murine progeria model. *Nat Commun* 2012;**3**:608.

194 J.M. Murphy, K. Dixon, S. Beck, *et al.* Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* 2002;**46**:704–713.

195 O.S. Beane, V.C. Fonseca, L.L. Cooper, *et al.* Impact of aging on the regenerative properties of bone marrow-, muscle-, and adipose-derived mesenchymal stem/stromal cells. *PLoS ONE* 2014;**9**: e115963.

196 M.L. Bustos, L. Huleihel, M.G. Kapetanaki, *et al.* Aging mesenchymal stem cells fail to protect because of impaired migration and antiinflammatory response. *Am J Respir Crit Care Med* 2014;**189**: 787–798.

197 E. Deak, B. Rüster, L. Keller, *et al.* Suspension medium influences interaction of mesenchymal stromal cells with endothelium and pulmonary toxicity after transplantation in mice. *Cytotherapy* 2010;**12**:260–264.

198 H. Yukawa, M. Watanabe, N. Kaji, *et al.* Monitoring transplanted adipose tissue-derived stem cells combined with heparin in the liver by fluorescence imaging using quantum dots. *Biomaterials* 2012;**33**:2177–2186.

199 X.B. Liu, H. Chen, H.Q. Chen, *et al.* Angiopoietin-1 preconditioning enhances survival and functional recovery of mesenchymal stem cell transplantation. *J Zhejiang Univ Sci B* 2012;**13**:616–623.

200 J. Gao, J.E. Dennis, R.F. Muzic, *et al.* The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells Tissues Organs* 2001;**169**:12–20.

201 C. Toma, W.R. Wagner, S. Bowry, *et al.* Fate of culture-expanded mesenchymal stem cells in the microvasculature: *in vivo* observations of cell kinetics. *Circ Res* 2009;**104**:398–402.

202 D. Furlani, M. Ugurlucan, L. Ong, *et al.* Is the intravascular administration of mesenchymal stem cells safe? Mesenchymal

stem cells and intravital microscopy. *Microvasc Res* 2009;**77**: 370–376.

203 D. Baksh, P.W. Zandstra, J.E. Davies. A non-contact suspension culture approach to the culture of osteogenic cells derived from a CD49e$^{low}$ subpopulation of human bone marrow-derived cells. *Biotechnol Bioeng* 2007;**98**:1195–1208.

204 E. Chavakis, C. Urbich, S. Dimmeler. Homing and engraftment of progenitor cells: a prerequisite for cell therapy. *J Mol Cell Cardiol* 2008;**45**:514–522.

205 J. Wu, Z. Sun, H.S. Sun, *et al.* Intravenously administered bone marrow cells migrate to damaged brain tissue and improve neural function in ischemic rats. *Cell Transplant* 2008;**16**:993–1005.

206 R.F. Pereira, K.W. Halford, M.D. O'Hara, *et al.* Cultured adherent cells from marrow can serve as long-lasting precursor cells for bone, cartilage, and lung in irradiated mice. *Proc Natl Acad Sci U S A* 1995;**92**:4857–4861.

207 S.G. Crich, L. Biancone, V. Cantaluppi, *et al.* Improved route for the visualization of stem cells labeled with a Gd-/Eu-chelate as dual (MRI and fluorescence) agent. *Magn Reson Med* 2004;**51**:938–944.

208 M. Widerøe, Ø. Olsen, T.B. Pedersen, *et al.* Manganese-enhanced magnetic resonance imaging of hypoxic–ischemic brain injury in the neonatal rat. *Neuroimage* 2009;**45**:880–890.

209 I. Yamada, S. Okabe, M. Enomoto, *et al.* Colorectal carcinoma: *in vitro* evaluation with high-spatial-resolution 3D constructive interference in steady-state MR imaging. *Radiology* 2008;**246**: 444–453.

210 C. Vandeputte, D. Thomas, T. Dresselaers, *et al.* Characterization of the inflammatory response in a photothrombotic stroke model by MRI: implications for stem cell transplantation. *Mol Imaging Biol* 2011;**13**:663–671.

211 C. Berger, M. Rausch, P. Schmidt, M. Rudin. Feasibility and limits of magnetically labeling primary cultured rat T cells with ferumoxides coupled with commonly used transfection agents. *Mol Imaging* 2006;**5**:93–104.

212 J.W. Bulte, D.L. Kraitchman. Iron oxide MR contrast agents for molecular and cellular imaging. *NMR Biomed* 2004;**17**:484–499.

213 E. Küstermann, U. Himmelreich, K. Kandal, *et al.* Efficient stem cell labeling for MRI studies. *Contrast Media Mol Imaging* 2008;**3**:27–37.

214 M. Hoehn, E. Küstermann, J. Blunk, *et al.* Monitoring of implanted stem cell migration in vivo: a highly resolved in vivo magnetic resonance imaging investigation of experimental stroke in rat. *Proc Natl Acad Sci U S A* 2002;**99**:16267–16272.

215 E.M. Shapiro, O. Gonzalez-Perez, J. Manuel García-Verdugo, *et al.* Magnetic resonance imaging of the migration of neuronal precursors generated in the adult rodent brain. *Neuroimage* 2006;**32**: 1150–1157.

216 J.P. Sumner, E.M. Shapiro, D. Maric, *et al. In vivo* labeling of adult neural progenitors for MRI with micron sized particles of iron oxide: quantification of labeled cell phenotype. *Neuroimage* 2009;**44**:671–678.

217 A. Crabbe, C. Vandeputte, T. Dresselaers, *et al.* Effects of MRI contrast agents on the stem cell phenotype. *Cell Transplant* 2010;**19**:919–936.

218 C. Belmar-Lopez, G. Mendoza, D. Oberg, *et al.* Tissue-derived mesenchymal stromal cells used as vehicles for anti-tumor therapy exert different *in vivo* effects on migration capacity and tumor growth. *BMC Med* 2013;**11**:139.

219 P. Walczak, J. Zhang, A.A. Gilad, *et al.* Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 2008;**39**:1569–1574.

220 J.A. Wood, D.J. Chung, S.A. Park, *et al.* Periocular and intra-articular injection of canine adipose-derived mesenchymal stem cells: an *in vivo* imaging and migration study. *J Ocul Pharmacol Ther* 2012;**28**:307–317.

221 A. Sohni, C.M. Verfaillie. Mesenchymal stem cells migration homing and tracking. *Stem Cells Int* 2013;**2013**:130763.

222 E.J. Sutton, T.D. Henning, S. Boddington, *et al. In vivo* magnetic resonance imaging and optical imaging comparison of viable and nonviable mesenchymal stem cells with a bifunctional label. *Mol Imaging* 2010;**9**:278–290.

223 L. Cussó, I. Mirones, S. Peña-Zalbidea, *et al.* Combination of single-photon emission computed tomography and magnetic resonance imaging to track $^{111}$In-oxine-labeled human mesenchymal stem cells in neuroblastoma-bearing mice. *Mol Imaging* 2014;**13**. doi: 10.2310/7290.2014.00033.

224 C.M. Deroose, S.K. Chitneni, R. Gijsbers, *et al.* Preliminary validation of varicella zoster virus thymidine kinase as a novel reporter gene for PET. *Nucl Med Biol* 2012;**39**:1266–1274.

225 E. Wolfs, T. Struys, T. Notelaers, *et al.* $^{18}$F-FDG labeling of mesenchymal stem cells and multipotent adult progenitor cells for PET imaging: effects on ultrastructure and differentiation capacity. *J Nucl Med* 2013;**54**:447–454.

226 S.C. Hung, W.P. Deng, W.K. Yang, *et al.* Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive *in vivo* positron emission tomography imaging. *Clin Cancer Res* 2005;**11**:7749–7756.

227 B.N. Park, J.H. Kim, K. Lee, *et al.* Improved dopamine transporter binding activity after bone marrow mesenchymal stem cell transplantation in a rat model of Parkinson's disease: small animal positron emission tomography study with F-18 FP-CIT. *Eur Radiol* 2015;**25** (5):1487–1496.

228 N. De Vocht, D. Lin, J. Praet, *et al.* Quantitative and phenotypic analysis of mesenchymal stromal cell graft survival and recognition by microglia and astrocytes in mouse brain. *Immunobiology* 2013;**218**:696–705.

229 I. Bergwerf, N. De Vocht, B. Tambuyzer, *et al.* Reporter gene-expressing bone marrow-derived stromal cells are immune-tolerated following implantation in the central nervous system of syngeneic immunocompetent mice. *BMC Biotechnol* 2009;**9**:1.

230 E.S. Kang, K.Y. Ha, Y.H. Kim. Fate of transplanted bone marrow derived mesenchymal stem cells following spinal cord injury in rats by transplantation routes. *J Korean Med Sci* 2012;**27**:586–593.

231 R.H. Lee, M.J. Seo, A.A. Pulin, *et al.* The CD34-like protein PODXL and α6-integrin (CD49f) identify early progenitor MSCs with increased clonogenicity and migration to infarcted heart in mice. *Blood* 2009;**113**:816–826.

232 I.A. Potapova, P.R. Brink, I.S. Cohen, S.V. Doronin. Culturing of human mesenchymal stem cells as three-dimensional aggregates induces functional expression of CXCR4 that regulates adhesion to endothelial cells. *J Biol Chem* 2008;**283**:13100–13107.

233 F. Zhang, Y. Hong, W. Liang, *et al.* Co-culture with Sertoli cells promotes proliferation and migration of umbilical cord mesenchymal stem cells. *Biochem Biophys Res Commun* 2012;**427**:86–90.

234 M. Shi, J. Li, L. Liao, *et al.* Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in

homing efficiency in NOD/SCID mice. *Haematologica* 2007; **92**:897–904.

235 S. Kumar, S. Ponnazhagan. Bone homing of mesenchymal stem cells by ectopic α4 integrin expression. *FASEB J* 2007;**21**:3917–3927.

236 R. Sackstein, J.S. Merzaban, D.W. Cain, *et al. Ex vivo* glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nat Med* 2008;**14**:181–187.

237 Z. Cheng, L. Ou, X. Zhou, *et al.* Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Mol Ther* 2008;**16**:571–579.

238 H.C. Ma, X.L. Shi, H.Z. Ren, *et al.* Targeted migration of mesenchymal stem cells modified with CXCR4 to acute failing liver improves liver regeneration. *World J Gastroenterol* 2014;**20**:14884–14894.

239 J.X. Yang, N. Zhang, H.W. Wang, *et al.* CXCR4 receptor overexpression in mesenchymal stem cells facilitates treatment of acute lung injury in rats. *J Biol Chem* 2015;**290**:1994–2006.

240 L.A. Marquez-Curtis, H. Gul-Uludag, P. Xu, *et al.* CXCR4 transfection of cord blood mesenchymal stromal cells with the use of cationic liposome enhances their migration toward stromal cell-derived factor-1. *Cytotherapy* 2013;**15**:840–849.

241 Y.W. Won, A.N. Patel, D.A. Bull. Cell surface engineering to enhance mesenchymal stem cell migration toward an SDF-1 gradient. *Biomaterials* 2014;**35**:5627–5635.

242 I.K. Ko, B.G. Kim, A. Awadallah, *et al.* Targeting improves MSC treatment of inflammatory bowel disease. *Mol Ther* 2010;**18**: 1365–1372.

243 D. Sarkar, P.K. Vemula, G.S. Teo, *et al.* Chemical engineering of mesenchymal stem cells to induce a cell rolling response. *Bioconjug Chem* 2008;**19**:2105–2109.

244 O. Levy, W. Zhao, L.J. Mortensen, *et al.* mRNA-engineered mesenchymal stem cells for targeted delivery of interleukin-10 to sites of inflammation. *Blood* 2013;**122**:e23–e32.

245 H. Yukawa, H. Noguchi, K. Oishi, *et al.* Cell transplantation of adipose tissue-derived stem cells in combination with heparin attenuated acute liver failure in mice. *Cell Transplant* 2009; **18**:611–618.

CHAPTER 23

# The role of chemokines in mesenchymal stromal cell homing to sites of inflammation, including infarcted myocardium

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## 23.1 Summary

A growing body of preclinical evidence suggests that mesenchymal stromal cells (MSCs) are effective for the structural and functional recovery of damaged organs, and numerous clinical trials have been, and are, underway using MSCs in a wide range of diseases. Systemic administration of MSCs has been used extensively in clinical studies, but the efficiency of culture-expanded MSCs homing and engraftment to the target organ is questionable. The therapeutic effect of MSCs in tissue repair and regeneration appears to correlate with the number of MSCs engrafted into the damaged tissue. Considerable progress has been made in the past decade in understanding the molecular mechanisms of the homing process of MSCs to a site of injury, particularly with regard to the involvement of chemokine receptors. This will ultimately help improve the homing ability of cultured-expanded MSCs and enhance their therapeutic effects.

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## 23.2 Introduction

Cell homing is a phenomenon that was initially discovered with hematopoietic stem cells (HSCs). HSCs are able to migrate through the bloodstream to various organs and return to their niche in the bone marrow under the guidance of chemical signals [1]. Bone-marrow-derived MSCs represent approximately 0.001–0.01% of all nucleated bone marrow cells and are approximately 10-fold less abundant than HSCs. It has been assumed that MSCs may exhibit a similar homing property. Clinical trials are underway in which bone marrow MSCs are administered into the blood, thereby eliminating the potential damage that could otherwise result from the use of intraorgan delivery routes [2,3]. However, studies have indicated that expansion of bone marrow MSCs *ex vivo* can severely impair their homing capacity [4], resulting in low levels of engraftment in target tissues [5–7]. Accordingly, there are concerns over the influence of poor engraftment affecting the outcome of MSC-based therapies.

In this chapter we provide an overview of the biology of MSC homing, focusing on the role of chemokine receptors and their ligands in MSC trafficking, migration, and engraftment at the site

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of tissue injury. We will also discuss the influence of culture expansion on the homing capacity and potentially the therapeutic effect of MSCs in tissue repair and regeneration.

## 23.3 Homing capacity of mesenchymal stromal cells

Several lines of evidence suggest that bone marrow MSCs have the ability to home to sites of tissue inflammation or damage. For an example, 24 h after transplantation of uncultured MSCs isolated from the bone marrow of transgenic mice that expressed enhanced green fluorescence protein (EGFP) into sublethally irradiated mice, as many as 55–65% of the $EGFP^+$ cells were recovered from the bone marrow and 4–7% were recovered from the spleen [4]. This high degree of homing to the marrow compares favorably with that of HSCs, of which approximately 25% returned to the bone marrow [8]. These results suggest that bone marrow MSCs exhibit a remarkable capacity for homing. A number of studies have indicated that bone marrow MSCs are capable of migrating from the blood into damaged tissues, crossing through endothelial cells to do so. When bone marrow MSCs were administered systemically after a stroke injury, the cells migrated toward the brain and were shown to acquire expression of the neural markers neuronal nuclear antigen, microtubule-associated protein 2, and glial fibrillary acid protein. The rats showed an improved functional outcome [9]. In another study using the ischemic stroke model, human MSCs were found in the damaged brain following ipsilateral injection (injected on same side as the stroke injury) or contralateral injection (injected on opposite side of the stroke injury) [10], indicating a capacity to home across the brain to the site of the stroke damage. Systemic infusion of bone marrow MSCs in baboons after lethal total body irradiation resulted in long-term engraftment of the cells in multiple organs, including the skin, intestines, liver, lungs, thymus, and kidney [11]. These results suggest that the homing and engraftment of bone-marrow-derived MSCs to injured tissues are independent of the cause of injury and/or the type of tissue.

Increasing evidence indicates that the homing capacity of bone marrow MSCs is severely impaired after *ex vivo* culture expansion [12]. When bone marrow MSCs were subjected to 24 h of *ex vivo* culture, the fraction of bone marrow MSCs detected in the bone marrow decreased to 10% compared with noncultured bone marrow MSCs. Likewise, after 48 h of culture, $EGFP^+$ bone marrow MSCs were not detected in lymphohematopoietic organs following systemic infusion [4]. We found that direct intramyocardial injection of allogeneic MSCs that had undergone five passages of monolayer culture into the ischemic area resulted in active migration of the cells through the ischemic region of the left ventricular wall and substantial engraftment (Figure 23.1), while only approximately 1–2% of systemically infused MSCs were detected in the acutely infarcted myocardium 72 h after intravenous infusion in mice (Figure 23.2). These

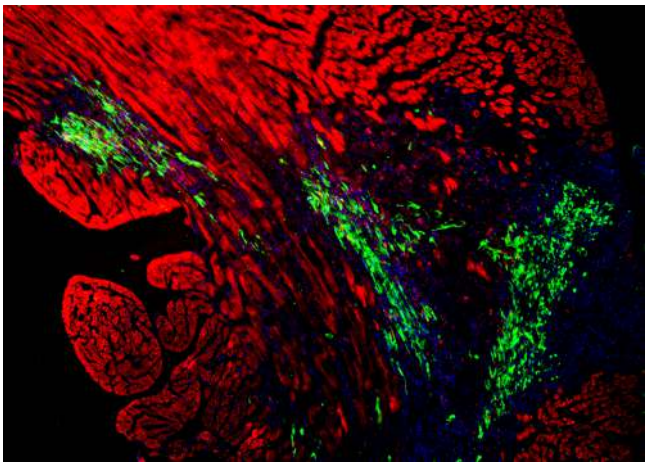


**Figure 23.1** MSC engraftment in the ischemic myocardium after intramyocardial injection in mice. A total of $0.5 \times 10^6$ bone-marrow-derived MSCs expressing green fluorescence protein were injected into the peri-infarct border zone at one site. After 72 h, MSCs (green) were found to have migrated through the ischemic areas from the injection site. Myocytes (red) were detected by antisarcomeric actin, and nuclei (blue) were stained with Hoechst. (See color plate section.)

results are consistent with other studies that also show low rates of engraftment of culture-expanded bone marrow MSCs into the ischemic myocardium following systemic infusion [13].

Studies have also indicated that culture-expanded MSCs injected intravenously lodge in the lungs and can cause vascular obstruction if sufficiently large numbers are administered. In

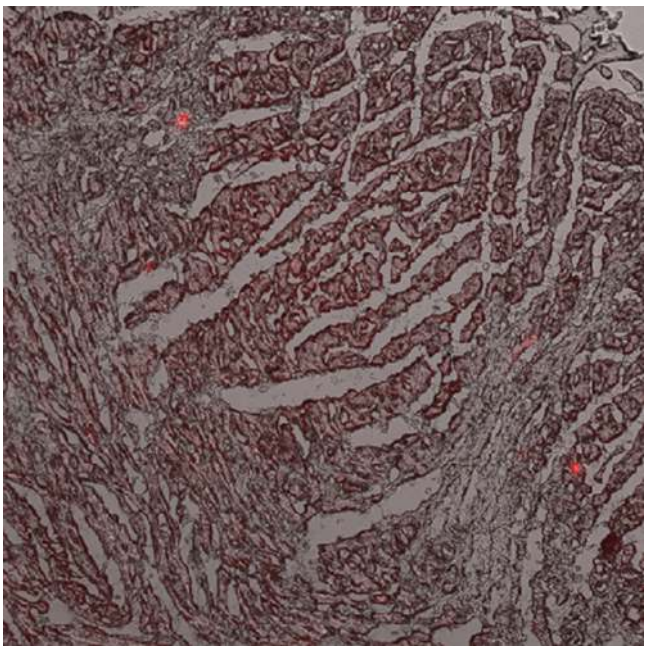


**Figure 23.2** Low engraftment of MSCs into the ischemic myocardium after systemic administration in mice. A microscopic image of a tissue section across the peri-infarct area of the acutely infarcted myocardium shows a few DiI-labeled MSCs (red) in the myocardum 72 h following intravenous infusion of $1 \times 10^6$ DiI-labeled MSCs. (See color plate section.)

several studies in mice over 80% of MSCs were trapped in the lungs following intravenous injection (Figure 23.3). These are likely stuck in the pulmonary capillary bed and appear to die of local ischemia [14–16].

Intra-arterial injection facilitates higher levels of cell engraftment in the target organ and may represent a more effective route for MSC administration [16–19]. However, intracoronary delivery of MSCs caused acute myocardial infarction (AMI) in dogs and reduction of coronary blood flow in pigs [13,20]. Similarly, intracarotid arterial infusion of MSCs caused a marked reduction in blood flow and the development of microstrokes in rats [21,22]. Thus, there is some concern over the safety of intravascular administration of MSCs. However, a meta-analysis and review of the literature by Lalu *et al.* that included over 1000 patients given human MSCs either intravenously or intra-arterially, of whom more than 300 were included in clinical trials, showed no adverse events related to the administration of the MSCs other than transient fever [23].

Bone marrow MSCs undergo dynamic changes in their molecular phenotype during culture expansion, although they may maintain stable expression of representative cell surface markers such as CD73, CD90, and CD105. Previous studies showed that human bone marrow MSCs in late passages lost surface expression of the chemokine receptors CCR1, CCR2, CCR7, CCR9, CXCR4, CXCR5, and CXCR6 together with chemotactic responses to their corresponding ligands [24,25]. Thus, it is necessary to optimize the culture condition for MSCs in order to maintain their properties, including the expression of homing receptors.

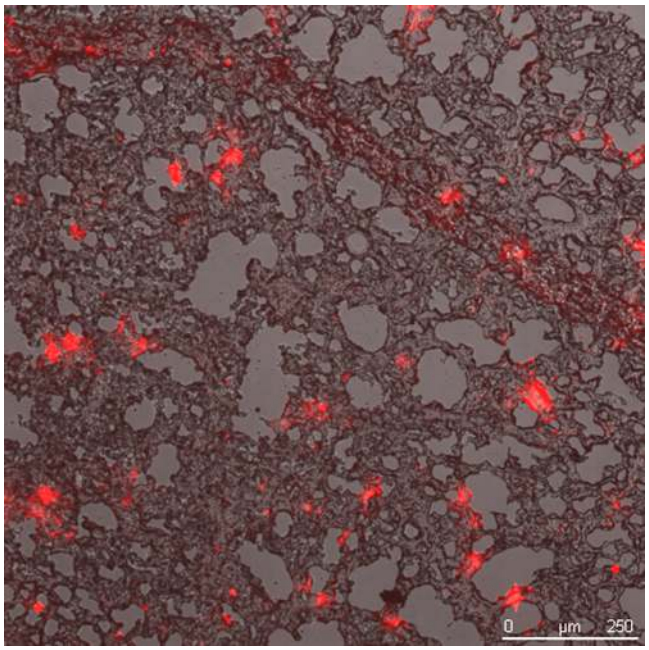



**Figure 23.3** Entrapment of MSCs in the lungs of mice. A microscopic image of a section of mouse lung tissue shows clusters of DiI-labeled MSCs (red) in the lungs 24 h after an intravenous injection of $0.5 \times 10^6$ DiI-labeled MSCs. (See color plate section.)

## 23.4 Homing ability of mesenchymal stromal cells and their therapeutic effects

Numerous animal studies have shown that direct delivery of MSCs into injured tissues can significantly enhance tissue repair [26–28]. Catheter delivery of allogeneic bone marrow MSCs to infarcted myocardium resulted in a profound reduction in scar formation and improvement in cardiac function in pigs [26]. Endocardial injection of bone marrow MSCs in pigs also showed cardioprotective effects in a model of chronic ischemic cardiomyopathy with significantly decreased fibrosis and improved left ventricular function [29]. Direct application of a monolayer sheet of MSCs on to the scarred myocardium resulted in reversed wall thinning in the scar area and improved cardiac function in rats [30].

However, comparative studies to evaluate the impact of different cell delivery routes on MSC engraftment rates and therapeutic effects are limited [3]. Three routes of bone marrow MSC administration have been used in preclinical models of cardiac disease: intramyocardial, intracoronary, and intravenous. Direct intramyocardial injection of bone marrow MSCs resulted in the highest rates of MSC engraftment and functional improvement of the left ventricle in animal AMI models [31,32]. In contrast, intracoronary infusion of bone marrow MSCs caused a reduction in coronary blood flow and microinfarction. Intravenous infusion of bone marrow MSCs resulted in a much lower rate of engraftment in the ischemic myocardium than either of the other two approaches [13]. Similarly, intracarotid administration of human MSCs led to significantly higher engraftment of MSCs in the injured brain of rats compared with intravenous transplantation [22,33]. However, whether low engraftment of intravenously infused MSCs to the target organ(s) ultimately compromises their therapeutic benefit needs to be clarified in parallel studies.

## 23.5 Mechanisms of leukocyte trafficking to sites of inflammation

Homing and engraftment is a prerequisite for cells to play their roles in the target tissue, particularly when cells are infused systemically [34]. The molecular mechanisms of homing of bone marrow MSCs have not been fully elucidated, but a preliminary study suggests that bone marrow MSCs exhibit some properties, such as rolling, similar to that adopted by leukocytes homing to inflammatory sites [35]. Thus, knowledge gained about leukocyte trafficking, adhesion, and migration may help in understanding the mechanisms mediating MSC homing. During inflammation, the recruitment of leukocytes to the inflammatory site requires a coordinated sequence of molecular and cellular events. The initial activity of leukocytes homing involves the rolling of the cells on the endothelium mediated by selectins, which are expressed in inflamed venules [36–39]. This process allows

leukocytes to become activated by cytokines and express high-affinity integrins on their surface, including CD18 and CD49d, which leads to the subsequent step of leukocyte arrest/firm adhesion on endothelial cells [40]. The transendothelial migration of leukocytes involves signals derived from clustering of apically disposed intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1), disassociation of endothelial junctions, and targeted recycling of platelet/endothelial cell adhesion molecules from the lateral border recycling compartment of endothelial cells [41]. Finally, the migration and invasion of leukocytes into the extracellular matrix (ECM) are mediated by integrin–ECM molecule interactions and matrix degradation [42].

## 23.6 Potential ligands/receptors for mesenchymal stromal cell homing

The molecular mediators involved with bone marrow MSC homing and engraftment into the site of tissue injury are not yet fully understood. Based on the hypothesis that specific chemoattractant molecules and adhesion molecules are upregulated in damaged tissue and that these induce the migration and engraftment of bone marrow MSCs, we developed a functional genomics strategy to identify candidate mediators for MSC homing. Genes that were significantly upregulated following AMI included CC chemokine ligands 2, 6, 7, and 9, chemokine CXC ligands 1, 2, and 12, interleukin (IL)-1 and IL-6, transforming growth factor (TGF)-β1 and -β2, tumor necrosis factor receptor II, and molecules involved in cell adhesion, including fibronectin 1, laminin 1, ICAM-1, VCAM-1, E-selectin, thrombospondin 1, and tenascin C [43,44]. These findings are in agreement with previous findings [45]. The expression of nine corresponding receptors of the upregulated ligands – namely, CCR2, CXCR4, IL-6RA, E-selectin ligand, CD29, CD49d, CD49f, CD49h, and CD49i – were detected in early passage murine bone marrow MSCs [43,44]. The functional involvement of some of these ligand–receptor pairs has been demonstrated.

Several additional ligand–receptor pairs have been reported that are possibly involved in MSC homing to sites of tissue injury. These include hyaluronic acid–CD44 [46], *N*-formylated peptides–*N*-formyl peptide receptor and the formyl peptide receptor-like-1 [47], platelet-derived growth factor (PDGF)-AB–PDGF-receptor alpha and beta, and insulin-like growth factor (IGF)-1–IGF receptor [48]. Further studies are required to establish their functional roles in MSC homing.

It still remains unclear whether MSCs utilize the same molecules for transendothelial migration as leukocytes do. Several molecules, such as CD18 and CD49d, which are critical for the trafficking and migration of leukocytes, are either not expressed or are expressed on the surface of a fraction of MSCs [44,49]. For leukocyte rolling along endothelial cells, the interactions of selectins with their corresponding ligands are considered essential [39]. Correspondingly, MSCs derived from bone marrow and placenta were observed to roll and adhere to postcapillary venules of a mouse model in a P-selectin-dependent manner [35]. However, it is controversial whether MSCs express functional selectin ligands, particularly after culture [50]. Nevertheless, culture-expanded bone marrow MSCs have been shown to exhibit rolling activity on activated endothelia and migrate to inflamed tissue after surface modification with a nanometer-scale polymer construct containing sialyl Lewis x [51]. Similarly, overexpression of CD49d in MSCs was shown to increase their homing to the bone marrow [52]. These studies suggest that adhesion molecules affect the homing capacity of MSCs.

## 23.7 Chemokine involvement in mesenchymal stromal cell homing

Currently, over 50 chemokines and 20 chemokine receptors have been discovered [53]. While most chemokines are secreted and are 67–127 amino acids in length, some chemokines are membrane bound (e.g., CXCL16 and CX3CL1). Chemokines are divided into four groups depending on the spacing of their first two cysteine residues near the NH2-terminus: CXC chemokines, CC chemokines, C chemokines, and CX3C chemokine [54].

Chemokine induction is a prominent feature in ischemic myocardium. Diverse chemokine/chemokine receptor axes participate in the regulation of chemotactic activities for various cell types. Following acute myocardial ischemia, several chemokines are upregulated immediately [43–45,55]. However, the upregulation is transient for many, and expression levels usually decline markedly within the first 72 h of acute AMI [43,44]. This chemokine expression pattern is consistent with the time course of homing of bone marrow MSCs. Freshly infarcted myocardium is more chemoattractive to bone marrow MSCs [56]. The expression of chemokine receptors in bone marrow MSCs and their corresponding ligands are summarized in Table 23.1. Notably, in a comparative analysis, similar expression levels of chemokine receptor proteins were found on the surface of bone marrow MSCs and MSCs derived from placenta, though differential display in mRNA expression level was found [50].

### 23.7.1 CCR1 and CCR2 involvement in mesenchymal stromal cell homing

A functional role for chemokines in the recruitment of bone marrow MSCs into infarcted myocardium has been implicated in several studies. CCL7, also known as monocyte chemoattractant protein (MCP), has previously been shown to attract monocytes and to regulate macrophage functions [62]. It has been found to be critically involved in bone marrow MSC homing to ischemic myocardium and in their intramyocardial migration and survival. Overexpression of CCL7 at the site of previous myocardial infarction recruited systemically administered bone marrow MSCs and improved cardiac structure and function in rats [56]. Consistently, increased expression of CCR1 (a receptor of CCL7) by murine bone marrow MSCs has been shown to

**Table 23.1** Chemokine receptor expression in bone-marrow-derived MSCs.

| Chemokine receptor | mRNA | | Intracellular protein | | Cell surface protein | | Ligands[a] | References |
|---|---|---|---|---|---|---|---|---|
| | Mouse | Human | Mouse | Human | Mouse | Human | | |
| CCR1 | +/– | + | N/A | +/– | N/A | 70%[b] | **CCL3**, **CCL5**, **CCL7**, **CCL8**, **CCL3L1**, **CCL13**, **CCL14**, **CCL15**, CCL16, CCL23 | [24,56–58] |
| CCR2 | + | + | + | +/– | +/– | – | **CCL2**, **CCL7**, **CCL8**, CCL13, CCL16 | [24,43,58–61] |
| CCR3 | + | + | N/A | +/– | 5% | – | **CCL5**, **CCL7**, **CCL8**, CCL11, CCL13, CCL15, CCL16, CCL24 | [24,56–58] |
| CCR4 | + | +/– | N/A | – | 12% | – | CCL17, CCL22 | [24,56–58] |
| CCR5 | + | + | N/A | +/– | +/– (rat) | – | **CCL5**, **CCL8**, CCL3, CCL4, CCL3L1 | [24,56–58] |
| CCR6 | – | +/– | N/A | +/– | 74% | – | CCL20 | [24,56–58] |
| CCR7 | + | + | N/A | +/– | 8% | 68% | CCL19,CCL21 | [24,56–58] |
| CCR8 | + | + | N/A | + | 4% | – | CCL1 | [24,56–58] |
| CCR9 | + | + | N/A | + | 43% | 59% | CCL25 | [24,56–58] |
| CCR10 | + | + | N/A | +/– | N/A | + | CCL27, CCL28 | [24,56–58] |
| CCR11 | N/A | + | N/A | – | N/A | +/– | CCL19, CCL21, CCL25 | [24,56–58] |
| CXCR1 | N/A | + | N/A | + | N/A | – | **CXCL8**, CXCL6 | [24,56–58] |
| CXCR2 | – | + | N/A | + | 25% | – | **CXCL1**, **CXCL2**, **CXCL8**, CXCL3, CXCL5, CXCL6, CXCL7 | [24,56–58] |
| CXCR3 | + | + | N/A | + | 55% | – | **CXCL10**, CXCL9, CXCL11 | [24,56–58] |
| CXCR4 | + | + | + | + | 0–27% | 0–45% | **CXCL12** | [24,56–58] |
| CXCR5 | – | + | N/A | + | 4% | 82% | CXCL13 | [24,56–58] |
| CXCR6 | + | + | N/A | + | 96% | 42% | CXCL16 | [24,56–58] |
| CX3CR1 | + (rat) | + | N/A | + | + (rat) | 20 ± 9% | **CX3CL1** | [24,56–58] |

–, not detected; +/–, weak expression; +, strong expression; N/A, data not available.
[a] Ligands in bold are upregulated by ischemia.
[b] Percentage of cells expressing the protein as determined by flow cytometry.

dramatically increase MSC chemotactic migration and to increase bone marrow MSC survival following intramyocardial delivery in mice [57]. Engraftment of bone marrow MSCs into the ischemic myocardium increased and was associated with an improved therapeutic effect.

CCR2 also appears to be a significant factor in the homing of bone marrow MSCs. MCP-1 (also known as CCL2, a ligand of CCR2) in the myocardium recruited murine bone-marrow-derived multipotent adult mesenchymal stromal cells (MASCs) administered systemically into the cardiac tissue [59]. MASCs used in the study represented a $CD34^{-}/Sca\text{-}1^{high}$ subpopulation of murine bone marrow cells that showed a high potential for differentiation and proliferation [59,63]. Following myocardial ischemia, expression of MCP-1 was upregulated immediately [43–45]. MASCs homed to regions of murine myocardium damaged by ischemia–reperfusion injury [59]. The cytoplasmic protein FROUNT has a pivotal function in CCR2-mediated monocyte chemotaxis. FROUNT, which interacts with CCR2 in the cytoplasm, appeared to be required for the effect observed with MASCs [59]. Together, these results indicate that myocardial overexpression of MCP-1 is associated with the recruitment of bone marrow MSCs to the myocardium in the absence of ischemic injury, suggesting a central role for MCPs in bone marrow MSC homing to the ischemic myocardium.

### 23.7.2 The CXCR4–SDF-1 axis in mesenchymal stromal cell homing

CXCL12 (SDF-1) has been shown to enhance the migration of uncultured bone marrow-derived hematopoietic stem cells (HSCs) to ischemic myocardium [64,65]. Because of these observations, it has been proposed that the CXCR4–SDF-1 axis also plays a role in bone marrow MSC homing. However, unlike HSCs, bone marrow MSCs express low levels of CXCR4 in a small subpopulation in early passages, and the expression levels decline progressively with successive passages [66,67]. The intracellular expression of CXCR4 appears to be sustained in bone

marrow MSCs in culture. Short-term exposure of human Flk1$^+$ bone marrow MSCs to a cocktail of cytokines consisting of Flt-3 ligand, stem cell factor (SCF), IL-6, hepatocyte growth factor (HGF), and IL-3 significantly upregulated both cell surface and intracellular levels of CXCR4, and this population of bone marrow MSCs was associated with an increase in migration capacity in response to SDF-1, as well as an increase in homing efficiency to the bone marrow of irradiated mice [68]. It is thus likely that the expression levels and distribution patterns of CXCR4 are regulated by cytokines. Similarly, exposing bone marrow MSCs to hypoxia increased their expression of CXCR4 and their migration to ischemic kidneys in mice [69]. In addition, forced expression of CXCR4 in cultured bone marrow MSCs resulted in an increase in their recruitment to acutely infarcted myocardium in rats [70] and to the site of traumatic brain injury in mice [71]. However, other studies have not confirmed these findings. In an acute kidney injury mouse model, separate and simultaneous overexpression of CXCR4 and CXCR7 (another receptor of SDF-1) in bone marrow MSCs did not increase their presence in the injured kidney [72]. Moreover, blockade of CXCR4 did not affect the intramyocardial migration of murine bone marrow MSCs to ischemic areas in mice [43]. These results suggest that the SDF-1–CXCR4 axis may be involved in the step of MSC extravasation but that the homing process requires the presence of other mediators. Preliminary data suggest that there is no correlation between serum SDF-1 levels and the number of circulating putative MSCs in patients with ischemic heart disease. For example, in patients with AMI an increase in blood SDF-1 levels was found to coincide with elevated levels of CD34$^+$ cells (normally present on human HSCs and endothelial cells) [73], but not of MSCs [71]. Thus, although the role of the CXCR4–SDF-1 axis in HSCs has been well recognized, the role of this signaling axis in the homing of bone marrow MSCs requires further study.

### 23.7.3 Other chemokines

Several other chemokines, including fractalkine (CX3CL1) and CCL25 (also known as thymus-expressed chemokine,), have been shown to influence MSC chemotaxis. Human bone marrow MSCs migrated in response to CCL25 in a dose-dependent manner [74]. CX3CR1 has been found to mediate both leukocyte migration and adhesion. Fractalkine expression is upregulated in the brain following ischemia in rats [75]. Hypoxic treatment of bone marrow MSCs increased their levels of CX3CR1 and their migration to fractalkine [76]. Knockdown of CX3CR1 expression in human bone marrow MSCs significantly reduced their recruitment to ischemic brain tissue after intravenous injection in rats [75]. These results suggest that fractalkine and CX3CR1 interactions are likely to play a role in the homing of bone marrow MSCs to sites of injured tissue. One study suggested that tissue-specific ligand–receptor pairs may be employed in the recruitment of bone marrow MSCs. As an example, the CCL27–CCR10 chemotactic axis showed the most potent effect in the recruitment of bone marrow MSCs to the skin. Increases in CCR10 expression in bone marrow MSCs or the level of CCL27 in the skin enhanced extravasation of MSCs from the circulation [25].

## 23.8 Pretreatment of mesenchymal stromal cells with cytokines and growth factors

Studies have suggested that the expression of certain chemokine receptors in MSCs can be upregulated after a brief exposure to cytokines and growth factors. PDGF-AB, TGF-β1, and tumor necrosis factor (TNF)-α showed potent chemoattractant activity *in vitro* to MSCs derived from human adipose, and these were more effective than SDF-1 or MCP-1. Pretreatment of MSCs with TNF-α significantly increased their chemotactic migration to several chemokines, such as RANTES, MCP-1, macrophage inflammatory protein 3β, and SDF-1 [77], suggesting an increase in the expression or function of the corresponding chemokine receptors. In an earlier study, exposure of human Flk1$^+$ bone marrow MSCs to a cocktail of cytokines consisting of Flt-3 ligand, SCF, IL-6, HGF, and IL-3 significantly upregulated both cell surface and intracellular levels of CXCR4 and increased their homing to the bone marrow [68]. In another study, the proinflammatory cytokines TNF-α, interferon-γ, IL-1α, and IL-6 significantly upregulated the mRNA and surface protein expressions of many chemokine receptors in murine bone marrow MSCs, including CCR2, CCR3, CCR5, and CXCR4. Moreover, MSCs pretreated with TNF-α exhibited significantly increased homing to inflamed skin or ischemic myocardium after intravenous injection into the retro-orbital sinus in mice [78]. These data suggest that priming of MSCs with proinflammatory cytokines before their administration may constitute an effective approach to enhance the homing of MSCs to sites of tissue injury.

## 23.9 Summary and future prospects

MSCs hold promise for improving the repair and regeneration of injured or diseased organs. However, the efficacy of this treatment appears to relate to the number of MSCs recruited to the target tissue. Therefore, the identification of homing mediators and the development of improved culture systems to maintain the homing capacity of MSCs are important. Chemokine receptors play a critical role in the homing of bone marrow MSCs to ischemic tissues, but many other mediators are likely involved in the complex homing process.

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## References


1 Lapidot, T., Dar, A., and Kollet, O. How do stem cells find their way home? *Blood* 2005;**106**:1901–1910.

2 Hare, J.M., Traverse, J.H., Henry, T.D., *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *J Am Coll Cardiol* 2009;**54**:2277–2286.

3 Salem, H.K., and Thiemermann, C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010;**28**: 585–596.

4 Rombouts, W.J. and Ploemacher, R.E. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* 2003;**17**:160–170.

5 Assis, A.C., Carvalho, J.L., Jacoby, B.A., *et al.* Time-dependent migration of systemically delivered bone marrow mesenchymal stem cells to the infarcted heart. *Cell Transplant* 2010;**19**:219–230.

6 Barbash, I.M., Chouraqui, P., Baron, J., *et al.* Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* 2003;**108**:863–868.

7 Kraitchman, D.L., Tatsumi, M., Gilson, W.D., *et al.* Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* 2005;**112**:1451–1461.

8 Vos, O., Luiten, F., and Ploemacher, R.E. Lodging of CFU(S) under various circumstances in bone marrow, spleen and liver. *Exp Hematol* 1980;**8**:860–866.

9 Chen, J., Li, Y., Wang, L., Zhang, Z., *et al.* Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001;**32**:1005–1011.

10 Kim, D., Chun, B.G., Kim, Y.K., *et al. In vivo* tracking of human mesenchymal stem cells in experimental stroke. *Cell Transplant* 2008;**16**:1007–1012.

11 Devine, S.M., Cobbs, C., Jennings, M., *et al.* Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003;**101**:2999–3001.

12 Karp, J.M. and Leng Teo, G.S. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* 2009;**4**:206–216.

13 Freyman, T., Polin, G., Osman, H., *et al.* A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. *Eur Heart J* 2006;**27**: 1114–1122.

14 Lee, R.H., Pulin, A.A., Seo, M.J., *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**:54–63.

15 Toma, C., Wagner, W.R., Bowry, S., *et al.* Fate of culture-expanded mesenchymal stem cells in the microvasculature: *in vivo* observations of cell kinetics. *Circ Res* 2009;**104**:398–402.

16 Fischer, U.M., Harting, M.T., Jimenez, F., *et al.* Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009;**18**:683–692.

17 Bliss, T., Guzman, R., Daadi, M., and Steinberg, G.K. Cell transplantation therapy for stroke. *Stroke* 2007;**38**:817–826.

18 Guzman, R., De Los Angeles, A., Cheshier, S., *et al.* Intracarotid injection of fluorescence activated cell-sorted CD49d-positive neural stem cells improves targeted cell delivery and behavior after stroke in a mouse stroke model. *Stroke* 2008;**39**:1300–1306.

19 Harting, M.T., Jimenez, F., Xue, H., *et al.* Intravenous mesenchymal stem cell therapy for traumatic brain injury. *J Neurosurg* 2009;**110**:1189–1197.

20 Vulliet, P.R., Greeley, M., Halloran, S.M., *et al.* Intra-coronary arterial injection of mesenchymal stromal cells and microinfarction in dogs. *Lancet* 2004;**363**:783–784.

21 Guo, L., Ge, J., Zhou, Y., *et al.* Three-dimensional spheroid-cultured mesenchymal stem cells devoid of embolism attenuate brain stroke injury after intra-arterial injection. *Stem Cells Dev* 2014;**23**:978–989.

22 Walczak, P., Zhang, J., Gilad, A.A., *et al.* Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 2008;**39**:1569–1574.

23 Lalu, M.M., McIntyre, L., Pugliese, C., *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012,**7** (10):e47559.

24 Honczarenko, M., Le, Y., Swierkowski, M., *et al.* Human bone marrow stromal cells express a distinct set of biologically functional chemokine receptors. *Stem Cells* 2006;**24**:1030–1041.

25 Alexeev, V., Donahue, A., Uitto, J., and Igoucheva, O. Analysis of chemotactic molecules in bone marrow-derived mesenchymal stem cells and the skin: Ccl27–Ccr10 axis as a basis for targeting to cutaneous tissues. *Cytotherapy* 2013;**15**:171–184. e1.

26 Amado, L.C., Saliaris, A.P., Schuleri, K.H., *et al.* Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A* 2005;**102**: 11474–11479.

27 Mangi, A.A., Noiseux, N., Kong, D., *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 2003;**9**:1195–1201.

28 Charwat, S., Gyongyosi, M., Lang, I., *et al.* Role of adult bone marrow stem cells in the repair of ischemic myocardium: current state of the art. *Exp Hematol* 2008;**36**:672–680.

29 Schneider, C., Krause, K., Jaquet, K., *et al.* Intramyocardial transplantation of bone marrow-derived stem cells: ultrasonic strain rate imaging in a model of hibernating myocardium. *J Card Fail* 2008;**14**:861–872.

30 Miyahara, Y., Nagaya, N., Kataoka, M., *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* 2006;**12**:459–465.

31 Perin, E.C., Silva, G.V., Assad, J.A., *et al.* Comparison of intracoronary and transendocardial delivery of allogeneic mesenchymal cells in a canine model of acute myocardial infarction. *J Mol Cell Cardiol* 2008;**44**:486–495.

32 Heldman, A.W. and Hare, J.M. Cell therapy for myocardial infarction: special delivery. *J Mol Cell Cardiol* 2008;**44**:473–476.

33 Lundberg, J., Sodersten, E., Sundstrom, E., *et al.* Targeted intra-arterial transplantation of stem cells to the injured CNS is more effective than intravenous administration: engraftment is dependent on cell type and adhesion molecule expression. *Cell Transplant* 2012;**21**:333–343.

34 Chavakis, E., Urbich, C., and Dimmeler, S. Homing and engraftment of progenitor cells: a prerequisite for cell therapy. *J Mol Cell Cardiol* 2008;**45**:514–522.

35 Ruster, B., Gottig, S., Ludwig, R.J., *et al.* Mesenchymal stem cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006;**108**:3938–3944.

36 McEver, R.P. Rolling back neutrophil adhesion. *Nat Immunol* 2010;**11**:282–284.

37 Imhof, B.A. and Aurrand-Lions, M. Adhesion mechanisms regulating the migration of monocytes. *Nat Rev Immunol* 2004;**4**:432–444.

38 Kamei, M. and Carman, C.V. New observations on the trafficking and diapedesis of monocytes. *Curr Opin Hematol* 2010;**17**:43–52.

39 Katayama, Y., Hidalgo, A., Furie, B.C., *et al.* PSGL-1 participates in E-selectin-mediated progenitor homing to bone marrow: evidence for cooperation between E-selectin ligands and α4 integrin. *Blood* 2003;**102**:2060–2067.

40 Hynes, R.O. Integrins: bidirectional, allosteric signaling machines. *Cell* 2002;**110**:673–687.

41 Muller, W.A. Mechanisms of transendothelial migration of leukocytes. *Circ Res* 2009;**105**:223–230.

42 Sorokin, L. The impact of the extracellular matrix on inflammation. *Nat Rev Immunol* 2010;**10**:712–723.

43 Ip, J.E., Wu, Y., Huang, J., *et al.* Mesenchymal stem cells use integrin beta1 not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell* 2007;**18**:2873–2882.

44 Wu, Y., Ip, J.E., Huang, J., *et al.* Essential role of ICAM-1/CD18 in mediating EPC recruitment, angiogenesis, and repair to the infarcted myocardium. *Circ Res* 2006;**99**:315–322.

45 Frangogiannis, N.G. Chemokines in the ischemic myocardium: from inflammation to fibrosis. *Inflamm Res* 2004;**53**:585–595.

46 Herrera, M.B., Bussolati, B., Bruno, S., *et al.* Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. *Kidney Int* 2007;**72**:430–441.

47 Viswanathan, A., Painter, R.G., Lanson, N.A., Jr, and Wang, G. Functional expression of *N*-formyl peptide receptors in human bone marrow-derived mesenchymal stem cells. *Stem Cells* 2007;**25**: 1263–1269.

48 Ponte, A.L., Marais, E., Gallay, N., *et al.* The *in vitro* migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities. *Stem Cells* 2007;**25**:1737–1745.

49 Pittenger, M.F., Mackay, A.M., Beck, S.C., *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**: 143–147.

50 Brooke, G., Tong, H., Levesque, J.P., and Atkinson, K. Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. *Stem Cells Dev* 2008;**17**:929–940.

51 Sarkar, D., Spencer, J.A., Phillips, J.A., *et al.* Engineered cell homing. *Blood* 2011;**118**:e184–e191.

52 Kumar, S. and Ponnazhagan, S. Bone homing of mesenchymal stem cells by ectopic α4 integrin expression. *FASEB J* 2007;**21**:3917–3927.

53 Bromley, S.K., Mempel, T.R., and Luster, A.D. Orchestrating the orchestrators: chemokines in control of T cell traffic. *Nat Immunol* 2008;**9**:970–980.

54 Lazennec, G. and Richmond, A. Chemokines and chemokine receptors: new insights into cancer-related inflammation. *Trends Mol Med* 2010;**16**:133–144.

55 Kollar, K., Cook, M.M., Atkinson, K., and Brooke, G. Molecular mechanisms involved in mesenchymal stem cell migration to the site of acute myocardial infarction. *Int J Cell Biol* 2009;**2009**:904682.

56 Schenk, S., Mal, N., Finan, A., *et al.* Monocyte chemotactic protein-3 is a myocardial mesenchymal stem cell homing factor. *Stem Cells* 2007;**25**:245–251.

57 Huang, J., Zhang, Z., Guo, J., *et al.* Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. *Circ Res* 2010;**106**:1753–1762.

58 Chamberlain, G., Wright, K., Rot, A., *et al.* Murine mesenchymal stem cells exhibit a restricted repertoire of functional chemokine receptors: comparison with human. *PLoS ONE* 2008;**3**:e2934.

59 Belema-Bedada, F., Uchida, S., Martire, A., *et al.* Efficient homing of multipotent adult mesenchymal stem cells depends on FROUNT-mediated clustering of CCR2. *Cell Stem Cell* 2008;**2**:566–575.

60 Ji, J.F., He, B.P., Dheen, S.T., and Tay, S.S. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stem cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* 2004;**22**:415–427.

61 Ringe, J., Strassburg, S., Neumann, K., *et al.* Towards *in situ* tissue repair: human mesenchymal stem cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 2007;**101**:135–146.

62 McQuibban, G.A., Gong, J.H., Tam, E.M., *et al.* Inflammation dampened by gelatinase A cleavage of monocyte chemoattractant protein-3. *Science* 2000;**289**:1202–1206.

63 Belema Bedada, F., Technau, A., Ebelt, H., *et al.* Activation of myogenic differentiation pathways in adult bone marrow-derived stem cells. *Mol Cell Biol* 2005;**25**:9509–9519.

64 Askari, A.T., Unzek, S., Popovic, Z.B., *et al.* Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy. *Lancet* 2003;**362**:697–703.

65 Abbott, J.D., Huang, Y., Liu, D., *et al.* Stromal cell-derived factor-1α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation* 2004;**110**:3300–3305.

66 Wynn, R.F., Hart, C.A., Corradi-Perini, C., *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* 2004;**104**:2643–2645.

67 Sordi, V., Malosio, M.L., Marchesi, F., *et al.* Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005;**106**:419–427.

68 Shi, M., Li, J., Liao, L., *et al.* Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. *Haematologica* 2007;**92**: 897–904.

69 Liu, H., Liu, S., Li, Y., *et al.* The role of SDF-1–CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stem cells for renal ischemia/reperfusion injury. *PLoS ONE* 2012;**7**:e34608.

70 Cheng, Z., Ou, L., Zhou, X., *et al.* Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Mol Ther* 2008;**16**:571–579.

71 Wang, Y., Johnsen, H.E., Mortensen, S., *et al.* Changes in circulating mesenchymal stem cells, stem cell homing factor, and vascular growth factors in patients with acute ST elevation myocardial infarction treated with primary percutaneous coronary intervention. *Heart* 2006;**92**:768–774.

72 Gheisari, Y., Azadmanesh, K., Ahmadbeigi, N., *et al.* Genetic modification of mesenchymal stem cells to overexpress CXCR4 and CXCR7 does not improve the homing and therapeutic potentials of these cells in experimental acute kidney injury. *Stem Cells Dev* 2012;**21**:2969–2980.

73 Wojakowski, W., Tendera, M., Michalowska, A., *et al.* Mobilization of CD34/CXCR4$^{+}$, CD34/CD117$^{+}$, c-met$^{+}$ stem cells, and mononuclear cells expressing early cardiac, muscle, and endothelial markers into peripheral blood in patients with acute myocardial infarction. *Circulation* 2004;**110**:3213–3220.

74 Binger, T., Stich, S., Andreas, K., *et al.* Migration potential and gene expression profile of human mesenchymal stem cells induced by CCL25. *Exp Cell Res* 2009;**315**:1468–1479.

75 Zhu, J., Zhou, Z., Liu, Y., and Zheng, J. Fractalkine and CX3CR1 are involved in the migration of intravenously grafted human bone marrow stromal cells toward ischemic brain lesion in rats. *Brain Res* 2009;**1287**:173–183.

76 Hung, S.C., Pochampally, R.R., Hsu, S.C., *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* 2007;**2**:e416.

77 Baek, S.J., Kang, S.K., and Ra, J.C. *In vitro* migration capacity of human adipose tissue-derived mesenchymal stem cells reflects their expression of receptors for chemokines and growth factors. *Exp Mol Med* 2011;**43**:596–603.

78 Kollar, K., Cook, M.M., Atkinson, K., and Brooke, G. Molecular mechanisms involved in mesenchymal stem cell migration to the site of acute myocardial infarction. *Int J Cell Biol* 2009;**2009**: 904682.

CHAPTER 24

# Live cell imaging and single cell tracking of mesenchymal stromal cells *in vitro*

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## 24.1 Introduction

Cells are highly dynamic biological systems that are comprised of a vast number of molecular processes that interact both spatially and temporally [1–3]. Researchers have access to many methods for studying cellular populations, though often these methods are not able to capture single cell behavior as well as rare or transient events [4,5]. Additionally, standard assays for studying cellular systems rely on snapshots of cell populations that do not reveal the true complexity and dynamics of single cell behavior [6]. Thus, continuous observations of single cells are necessary to accurately study how the fate of an individual cell is adopted in culture and how that fate is related to the cell's molecular state (Figure 24.1). For mesenchymal stromal cell (MSC) cultures, which are heterogeneous and may only contain a small population of stem cells, single cell analyses are even more critical [7,8]. This is because MSC populations are comprised of various cell types that exhibit differential growth potential and potency [9]. Population averages do not reveal the underlying detail of complex systems and often lead to incorrect conclusions [6,10] (Figure 24.1a and c). Thus, to accurately study MSC cell growth dynamics (division, apoptosis, self-renewal, and lineage commitment) it is critical to continuously observe each cell throughout its life cycle and record lifetime events [10] (Figure 24.1b).

Interestingly, the importance of continual observations of biological processes was first recognized in the 17th century by Leeunwenhoek when observing capillaries in the ears of living rabbits using the first microscopes [11]. In the 19th and 20th centuries the development of time-lapse photography, fluorescent microscopy, and improvements in cell culture techniques enabled continuous observation of molecules within living cells over long periods of time [4]. The field of live cell imaging has grown exponentially since this time, driven by the need to continuously observe cell fate outcomes in lineage-tracing and fate-mapping experiments in developmental biology research. In the 21st century, live cell time-lapse imaging has been successfully applied to understanding questions in

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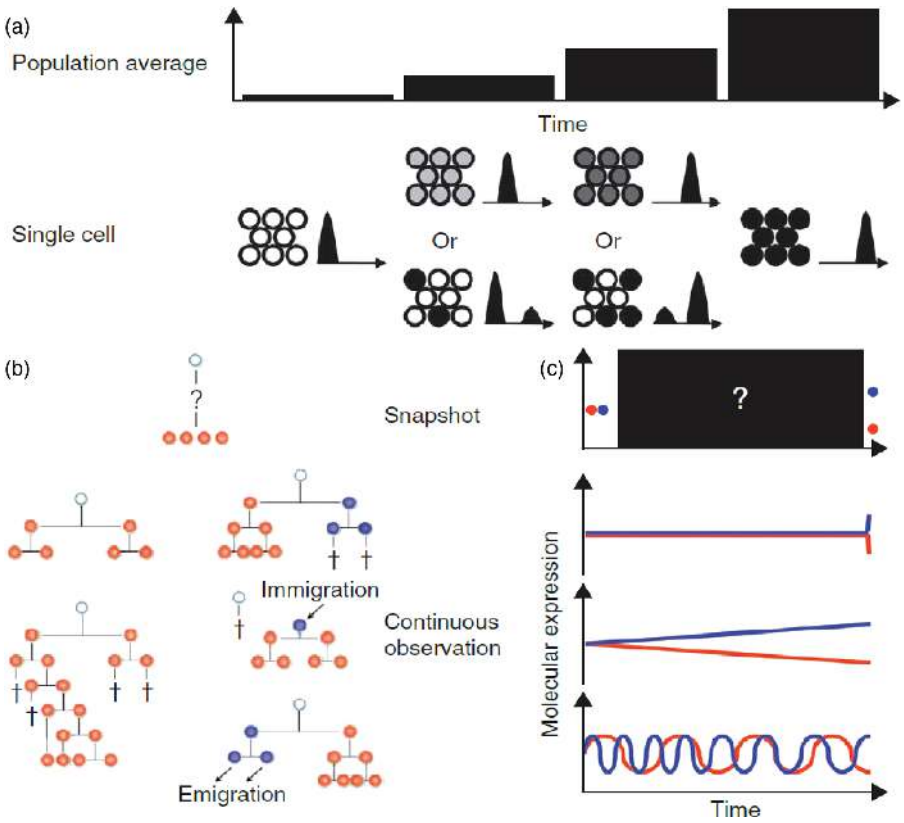



**Figure 24.1** Continuous observation of single cells versus population averages. (a) Population averages obscure population heterogeneity as determined by flow cytometry or single cell analyses. (b) Continuous observation of single cells is required to understand cell fate in detail. There are many different possibilities for how heterogeneous populations arise. For example, the only way to know how one white cell can give rise to four cells is to continuously observe each cell's fate. (c) Extrapolating from average population-based measurements of molecular expression to single cells can lead to erroneous conclusions. (See color plate section.) *Source:* [6] Schroeder 2011, pp. S30–S35. Reproduced with permission of Nature Publishing Group.

MSC biology that would otherwise have been impossible. These include, but are not limited to, characterizing the onset of heterogeneity in expanded MSCs [12], identifying a new population of MSCs in the endometrium [13], studying MSC spreading, proliferation and migration in response to chemically defined substrates [14], soluble and fixed gradients, direct current electric fields [15], and bioengineered scaffolds [16]. Indeed, there have been a greater number of studies applying live cell imaging to addressing hematopoietic, neural, and immune cell populations compared with those studying MSC populations. The most likely explanation for this is that these cellular systems have well-developed markers to track developmental stages and lineage progression. Researchers wishing to apply live cell imaging to answer questions in MSC biology will learn from other cellular systems. Examples include demonstrating blood cell generation from hemogenic endothelium [17] (hemogenic endothelium is a special subset of endothelial cells (ECs) scattered within blood vessels that can differentiate into hematopoietic cells), studying neural stromal cell progression [18], identifying new features of self-renewal in hematopoietic stromal cells [19], proving that hematopoietic cytokines are instructive and not selective [20], modeling intracellular competition for cell fate in B lymphocytes [21], and developing new models that describe lymphocyte cell cycle kinetics [22].

Live cell imaging is a multidisciplinary area of research that has evolved through input from engineers, physicists, chemists, biologists, computer scientists, and mathematicians. This level of technical sophistication can be a barrier to entry for some laboratories where there is no access to expertise from these different disciplines. Furthermore, there is not a standard protocol for live cell imaging because imaging parameters depend on the type of tracked cells and the hypothesis to be tested [4]. Thus, the great challenge is to make this technology more accessible to the biology community. This chapter will provide an in-depth understanding of the technical considerations for optimal time-lapse imaging and analysis of MSC fate.

A typical workflow involves (i) image acquisition, (ii) image processing, (iii) cell tracking, annotation of pedigrees, and (iv) quantification and visualization (Figure 24.2). Implementation of this workflow requires careful choice of imaging modalities, cell culture environments, imaging acquisition parameters (time-lapse interval, image illumination time, and experiment duration), experimental design, data storage, image processing, and software for cell tracking and annotation. Imaging parameters must be based upon the cell type and biological hypothesis that is being tested [4]. This chapter first describes technical considerations for image acquisition, process and storage (Section 24.2), methods for single cell tracking and analysis (Section 24.3) and a case study examining differentiation of ECs from cardiac-derived MSCs (Section 24.4) [23].

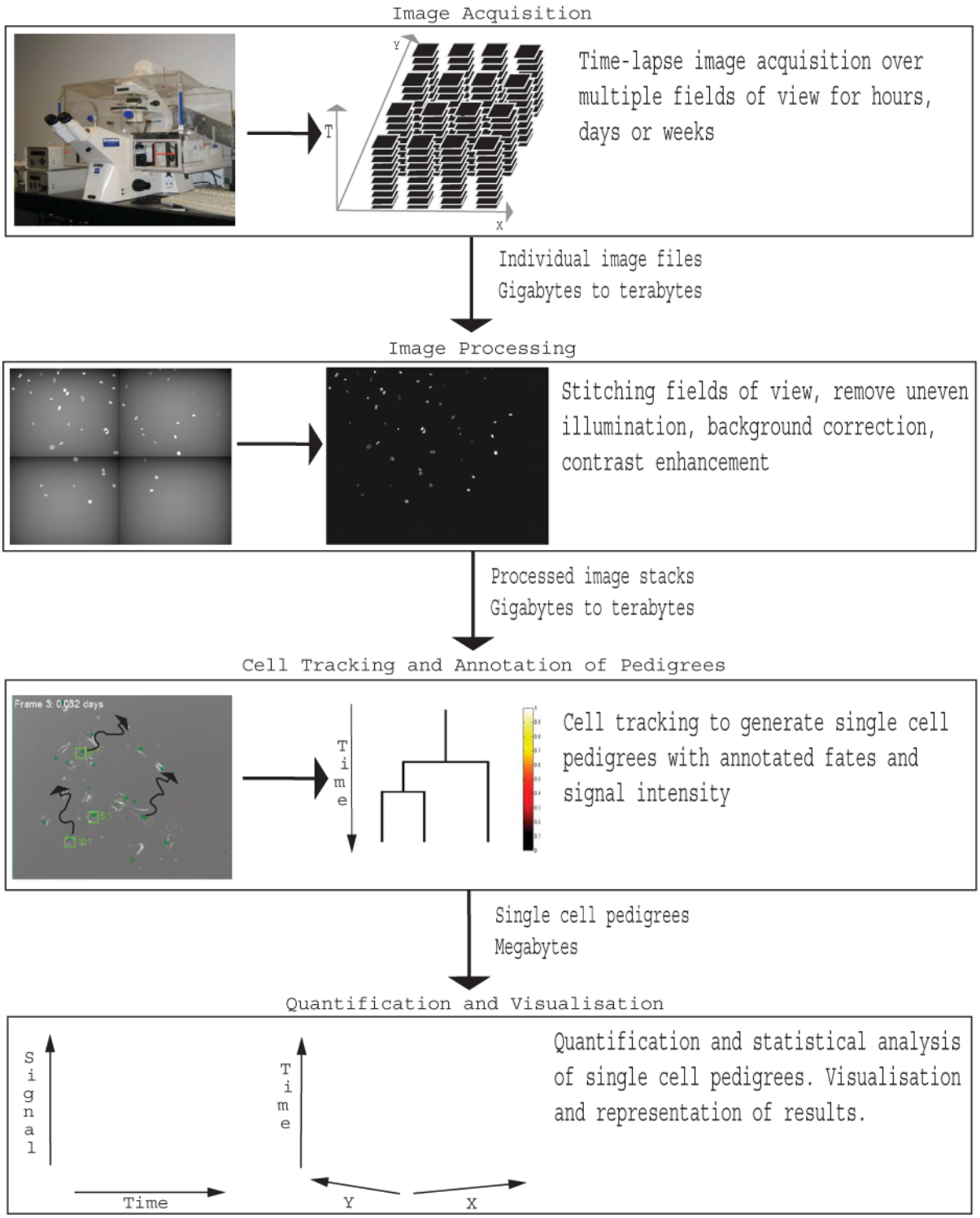



**Figure 24.2** Generic workflow for live cell imaging and cell tracking. Image acquisition is used to generate time-lapse images over multiple fields of view. Image processing is required to enhance image quality for cell tracking and quantification. Processed image stacks are then analyzed by specialized software that identifies cell trajectories from which single cell pedigrees are built. These trajectories can be annotated with discrete cell fate outcomes or continuous measurements, such as fluorescence or cell size. Finally, cell fate outcomes are visualized using specialized software with appropriate statistics to test hypotheses.

## 24.2 Technical considerations

### 24.2.1 Equipment, software, and hardware requirements

Specially designed equipment, hardware, and software are required to maintain cell viability and allow continuous observations of cells in more than one field of view (FOV) over the long term (several days or more). The hardware components of a live cell imaging system include (a) an automated inverted fluorescent microscope with a motorized *X–Y–Z* stage controller, transmitted and fluorescent light sources, and appropriate fluorochrome excitation and emission optics, (b) an environmental chamber with temperature regulation and respiratory gas controller ($CO_2$ with optional $O_2$ control), (c) a scientific complementary metal-oxide-semiconductor or charge-coupled device high-sensitivity digital camera, and (d) a computer for time-lapse image storage and programmatic control of all hardware elements [6]. Custom-written or commercially available software is required to define image acquisition parameters, control the microscope, and review data. The primary objective of a live cell imaging experiment is to maintain cell viability on the microscope stage and prevent abnormal cell growth that can arise from overexposure to light (phototoxicity) that would result in experimental artefacts [24].

Controlled temperatures are achieved by coupling of an environmental chamber to a heating unit and temperature controller that together are able to maintain the specimen at a specific temperature, typically 37 °C. In most arrangements a small temperature probe is used to continuously measure temperature as close to the sample as possible. Real-time temperature measurements are fed into a heating controller that adjusts the power of the incubator heater to maintain the sample environment at the temperature set-point. Environmental chambers either enclose the entire microscope or just the culture plates that sit on top of the microscope stage. There is often a few degrees of temperature variation within large environmental chambers; hence, there is a need for the temperature sensor to be as close to the sample as possible.

Environmental chambers are typically "leaky," so control of respiratory gases is potentially problematic. To achieve a stable pH for bicarbonate/$CO_2$-buffered culture systems, dedicated $CO_2$ controllers mix a $CO_2$ supply with air and pump the mixture through a humidification bottle and into the environmental chamber. For incubators that enclose the microscope, a secondary cover encloses the culture vessel through which respiratory gases are flowed. Humidification of the supplied gas mixture is necessary to reduce evaporation from the culture vessel, a particularly important consideration when using small-volume cell culture systems (e.g., microfluidics). While 5% $CO_2$ is commonly used for the buffering pH in standard tissue culture incubators, there are several suitable alternatives that do not require $CO_2$ control, such as HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer.

Open chambers that surround the entire microscope stage are suitable for experiments using large petri dishes or multiwell plates as they provide more accessibility to the sample; for example, for microinjection, single-cell capture, addition of factors, or exchange of medium. Closed small-volume chambers that sit upon the microscope stage are a cheaper option, providing a more isolated and regulated environment surrounding the specimen but without any space to access the specimen.

Maintaining image focus is crucial for cell tracking, particularly for experiments over several days or weeks, where microscope components inevitably drift with time. Autofocus algorithms are required to maintain image focus. Software algorithms that are based on multiple captured images are slow and unreliable in comparison with hardware autofocus, which uses reflection of a light beam off the glass bottom of the culture vessel to maintain a constant optical path length between the sample and microscope lens. Hardware autofocus only works with high magnification objectives (10× or higher) and requires a focal plane to be set manually, which can increase set-up time if acquiring a large number of FOVs. However, even though software autofocus does not require additional set-up time, one must consider that acquisition of multiple images for each focus position will increase stage scanning time and potentially contribute to phototoxicity and fluorochrome photobleaching.

The quality of commercial systems for live cell imaging has improved dramatically over the last 5 years. These are available from suppliers such as Leica, Nikon, Olympus, Molecular Devices, and Zeiss. The Incucyte™ live cell imaging system (Essen Biosciences) departs from the standard configuration by microscopically scanning culture plates within an incubator. The major difference between these platforms relates to their programmatic and optical imaging flexibility.

Transmission light microscopy using phase contrast or differential interference contrast (DIC) is preferably used for cell tracking as it does not require much light to obtain an image [25]. Phase-contrast microscopy is able to convert optical path differences generated as light passes through a specimen into contrast differences in the object image. DIC is similar to phase contrast: the major difference is that DIC microscopy converts gradients in the optical path length as light passes over the specimen into regions of contrast in the object image. DIC microscopy produces images that appear almost three-dimensional and does not result in phase halos as with phase contrast.

Transmitted-light microscopy does not require significant levels of illumination, enabling more frequent visualization of cell ultrastructure without phototoxicity. Therefore, dynamic cellular process, such as division, death, and changes in cell ultrastructure, can be tracked in more detail. Additionally, this information is obtained without the use of fluorescent probes or dyes, which require phototoxicity studies to ensure that cell growth dynamics are not perturbed [26].

Fluorescent imaging modalities are required to obtain molecular information. Individual fluorescent molecules fluoresce when excited at a particular wavelength of light. The location

and dynamic movement of fluorescently labeled molecules can be used to mark specific subcellular compartments, cytoskeletal structures, or individual proteins themselves. Common fluorescent probes for directly labeling subcellular compartments include Hoeschst dye that stains DNA, and fluorescently labeled phalloidin that marks cytoplasmic actin filaments. Fluorescent proteins can also be used as reporters of gene expression within living cells. Section 24.3.3 examines the use of fluorescent biomarkers for live cell imaging of MSCs in more detail.

Some of the modalities for live cell fluorescent imaging are briefly described as follows:

- **Fluorescence recovery after photobleaching (FRAP):** FRAP can be used to estimate diffusion coefficients and dynamics of protein binding processes. FRAP provides an optical record of dynamic cellular interactions with high spatiotemporal resolution. For example, FRAP has been used to study the process of cellular coupling in a co-culture of quantum dot (QD)-labeled MSCs and cardiomyocytes [27].
- **Fluorescence energy transfer (FRET):** excitation of a shorter wavelength fluorochrome results in fluorescence of a longer wavelength fluorochrome if the two fluorochromes are within a particular molecular distance of each other. FRET can be used to study the interaction between neighboring proteins or interaction sites. For example, FRET has been used to visualize the activity of FAK and Src signaling pathways at the plasma membrane in human MSCs during stimulation of osteogenic, myogenic, and neurogenic differentiation pathways [28].
- **Total internal reflection (TIRF) microscopy:** induction of an evanescent wave in a limited area surrounding a region of interest in a specimen that is immediately adjacent to two mediums with different refractive indices. Typically, the two mediums used to generate the evanescent wave are the specimen and a glass coverslip. For example, TIRF has been used to visualize enrichment of guanine nucleotide exchange factor H1 in MSC focal adhesions after osteogenic induction [29]. Interestingly, inhibition of focal adhesion formation in MSCs resulted in suppression of osteogenic commitment, suggesting a role for focal adhesion signaling in the regulation of MSC fate decisions.

There are many detailed reviews on fluorescent imaging methods, including those already listed and others [25,30–32]. When these methods are applied to tracking single cell molecular processes one will need to optimize the trade-off between the amount of image information achieved, which usually correlates with the quantity of illumination, and phototoxicity or photobleaching.

### 24.2.2 Image acquisition parameters

Image acquisition parameters must be defined for each live cell imaging experiment. Parameters will be adjusted depending on the cell type being studied, cell culture density, use of fluorescent reporters, and the type of dynamic responses being observed. These parameters include image acquisition frequency, the length of observation period, exposure time, and the number of FOVs per time lapse. Image acquisition frequencies of the order of seconds to minutes are suitable for capturing fast processes, such as the detailed observation of phases of mitosis or intracellular vesicle trafficking. Dynamic interactions of MSCs with other cell types, such as their transmigration between and directly through ECs, requires image acquisition every 5–90 s to gain insight into the critical ability of MSCs to extravasate at sites of inflammation [33]. Furthermore, the mechanisms that MSCs use to interact with ECs and cancer cells require image acquisition every 1 min so that they can be visualized [34]. A temporal resolution of the order of several minutes to half an hour is necessary to resolve movement of individual cells in consecutive frames when tracking cells that are highly motile, or are a part of a dense culture, where cells frequently make contact. MSC cultures are highly proliferative and migratory and they can form dense colonies where cells frequently touch and move over the top of each other. Therefore, in most instances an image acquisition interval of 5–20 min is most suitable for accurately tracking individual cells in an MSC culture. At the other end of the temporal scale, an image acquisition frequency of 60 min or more would be useful for tracking cells that are not motile and do not divide rapidly. Alternatively, if studying the migration of a population of MSCs, such as in a wound healing assay where the time to wound closure is measured, a temporal resolution of 1–2 h can be sufficient [35]. Acquisition of fluorescent images at high temporal frequency can result in phototoxic effects, and therefore exposure to excitation light should be minimized. This can be achieved by reducing exposure times or setting fluorescent image acquisition to the lowest possible temporal resolution that still captures the process of interest. Image acquisition frequency determines the number of sites that can be imaged for each time lapse. If images are acquired every 15 min, then the maximum number of sites is determined by how quickly the microscope travels to and acquires images at each site. The speed of image acquisition is also dependent on the use of software or hardware autofocus and the number of image modality channels.

The duration of an experiment in some cases can be difficult to define without analysis of pilot experiments. When recapitulating a standard *in vitro* assay (for example, a colony-forming assay or differentiation assay) the duration of the assay is well known. However, it is not possible to estimate generation times or differentiation dynamics directly from bulk culture experiments or clonogenic assays [20]. A trial time-lapse imaging experiment will provide an indication of phototoxicity, division times, and photobleaching effects before large-scale experiments are planned.

### 24.2.3 Image processing

Image restoration and enhancement methods are required to improve the accuracy of cellular tracking and fluorescent quantification. Uneven illumination, though not obvious to the human eye, will add spatial bias to fluorescent measurements. An image restoration algorithm is required to return the image to even illumination so that cellular fluorescent measurements do not

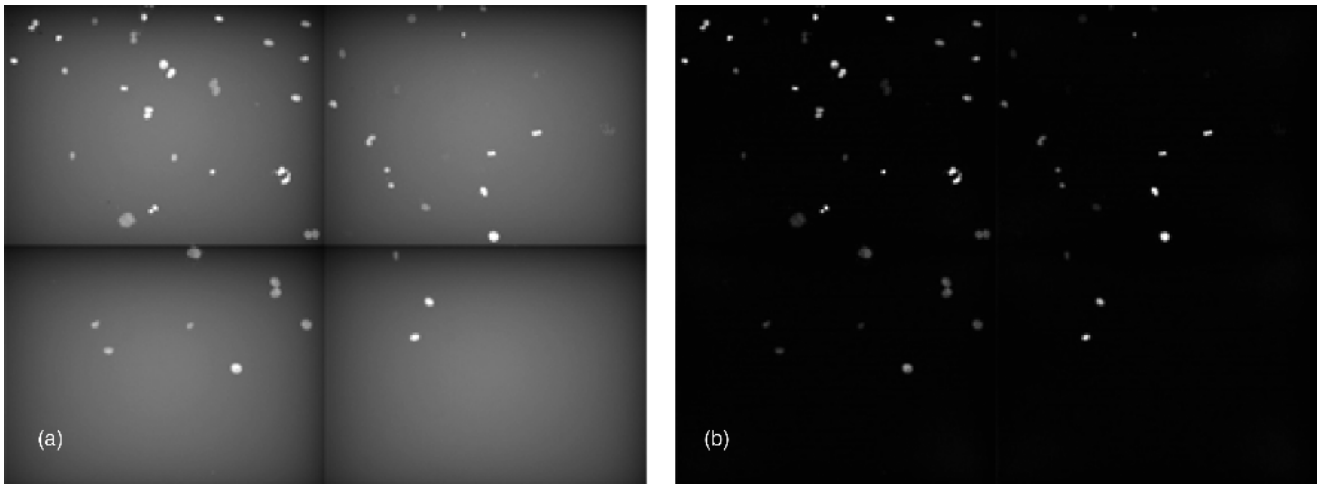



**Figure 24.3** Fluorescent images showing nuclear expression of platelet-derived growth factor receptor alpha (PDGFR-α) in cardiac MSCs before and after image processing. Four FOVs are stitched together to form one larger FOV for cell tracking. (A) Fluorescent image shows raw images after stitching and (B) the final result after correction of uneven illumination and contrast. (350 ms exposure time.)

depend on their position in the microscopic FOV. Another image restoration method that is commonly applied for wide-field imaging using multiple contiguous FOVs is image stitching. The edges of overlapping images must be registered so that the composite image appears as one image. Image registration applied to consecutive images is sometimes required to eliminate sample jitter caused by backlash in translational stage movements.

Image enhancement methods aim to accurately segment cells from their background. The toolbox of image enhancement methods includes algorithms to enhance image contrast and segmentation methods such as image thresholding, morphological operations (opening and closing), and edge detection. Figure 24.3A shows an unprocessed fluorescent image of four adjacent fields of view stitched together. Four fields of view were stitched together so that cells could be tracked in a large FOV. The fluorescent images are of a population of cardiac MSCs expressing a nuclear GFP reporter for PDGFR-α. Uneven illumination over the FOV results in a higher background reading at the center of the image. Figure 24.3B shows the same image after correction of uneven illumination, subtraction of background, and enhancement of contrast with custom-written scripts using the Matlab™ image processing toolbox.

Commercial packages such as MetaMorph (Molecular Devices), Imaris (Bitplane), PhotoShop (Adobe), and Amira (VSG) also meet these requirements and typically offer different algorithms so that image processing can be optimized. Alternatively, there are free and open-source image processing software packages available such as ImageJ and Fiji. Open-source software offers several advantages, including the addition of plug-ins developed and validated by the imaging research community. With open-source packages researchers are able to write their own plug-ins, suitable for optimization or specialized requirements. Engineering programming platforms such as Matlab can also be used to implement an image processing pipeline. Matlab has comprehensive functionality packaged into image processing and computer vision toolboxes.

Image segmentation is a commonly used image processing tool to measure morphological features such as cell size and shape. Automated cell tracking algorithms such as CellProfiler only work with segmented images. The complex morphological characteristics of MSCs should be considered when applying image segmentation algorithms to ensure individual cells are accurately segmented. MSCs can appear morphologically as small and spindle shaped, stellate, or large and flat. MSCs can also have many cellular projections, such as philopodia and lamellapodia. An example of image segmentation of MSCs using CellProfiler is shown in Figure 24.4. Figure 24.4A shows MSC image segmentation at low density and Figure 24.4B shows image segmentation of subconfluent MSCs after 5.75 days in culture, with cells color coded by their generation number.

### 24.2.4 Data storage

Live cell imaging experiments, particularly long-term imaging experiments, have the potential to generate a large volume of image data. For example, consider an experiment in which 300 positions were imaged with phase contrast every 10 min and with two fluorescent channels every 60 min for a total of 9 days. Assuming images were acquired as high-resolution TIFF files, each occupying 2.5MB, the volume of data generated would exceed 500GB. Without access to dedicated data storage architecture, long-term storage of large amounts of data can be challenging. At present, internal hard disk drives (HDDs) for desktop computers are available up to 2TB and so can only hold a few large experimental data sets. Researchers at universities and research institutions can have access to large servers to store and backup terabytes of data although such services do not always have network speeds suitable for fast data recovery. While data are being analyzed it should be accessible and retrievable at a

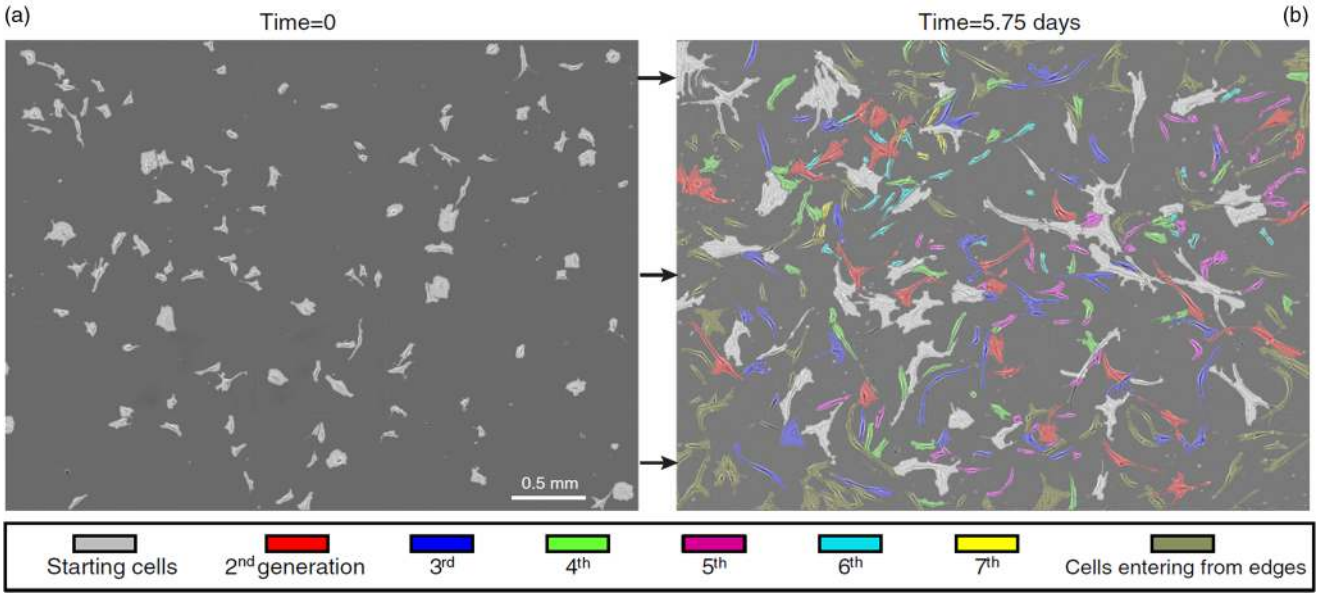



**Figure 24.4** Image segmentation of MSCs at the beginning and end of a single passage (p5). Phase-contrast image of entire FOV at (A) the beginning and (B) the end (5.75 days) of the experiment with cells false colored to represent their generation as indicated. All cells at time zero were classified as starting cells that produce two second-generation daughter cells upon division. (See color plate section.) *Source:* [12] Whitfield *et al.*, 2013, pp. 1365–1377. Reproduced with permission of Elsevier.

reasonable speed. In the absence of large institutional servers, by far the simplest and cheapest option is to store data on external HDDs. Current external HDDs have a capacity to store 4TB, although external HDDs eventually fail, so a data backup system will be required. Therefore, it is recommended that researchers use a combination of technologies to maintain the fidelity of their data over the long term [36].

## 24.3 Single cell tracking and analysis

### 24.3.1 Cell tracking platforms

Time-lapse imaging experiments generate image stacks for individual FOVs over a period of hours, days, or weeks. A single cell tracking platform and an analysis pipeline are required to record the characteristics of each cell as seen in consecutive images within a time-lapse movie. The fate of each cell, be it division, death, or right censoring (lost or not observed), provides the genealogical information to construct single cell pedigrees (Figure 24.5). Pedigrees are annotated with multiple single cell measurements, such as morphological features, fate outcomes, and fluorescent intensity. An early application for single cell tracking was to prove the existence of specific cell fate transitions such as the existence of hemogenic endothelium *in vitro* [17]. Single cell pedigree information also yields important dynamic and genealogical data, which is complementary to bulk culture and clonogenic observations.

Presently, researchers use a combination of propriety software, open-source software, or custom-written algorithms for cell tracking and pedigree analysis. The most significant analysis bottleneck is cell tracking. A massive effort is underway to develop universal solutions that will improve upon "point-and-click" tracking. The following section reviews a range of cell tracking platforms and discusses their specific advantages and disadvantages.

Commercially available cell tracking software has been developed by companies such as Cellomics, Evotec, and Molecular Devices. These platforms were developed for high-throughput screening of cellular responses to pharmacological and toxicological agents. The systems were designed for detecting only relatively specific cellular responses, and not continuous cell trajectory tracking. Protein translocation, microvesicle formation, neurite growth, cell count, and viability have been screened with such systems [37]. More flexible and cost-effective tracking platforms have been implemented in a number of different image analysis packages, such as MetaMorph, ImagePro, ImageJ, and Fiji (Box 24.1). Such platforms offer automation and are suitable for researchers with basic requirements, including cell identification, cell trajectory measurements, and track creation. For example, MetaMorph's TrackObject has been used to study the migration of MSCs on polydimethylsiloxane (PDMS) substrates with 350 nm grating topographies. In this study, MSCs cultured on PDMS with such nanotopographies had faster and more directed migration [38]. The change in migration rate was related to a downregulation in zyxin protein that resulted in more dynamic and smaller focal adhesions. Zyxin may function as a messenger in the signal transduction pathway that mediates adhesion-stimulated changes in gene expression and may modulate the cytoskeletal organization of actin bundles. The ImageJ plug-in MTrackJ was used to study the effect of co-culture with dendritic cells on MSC motility [39]. Co-culture reduced both

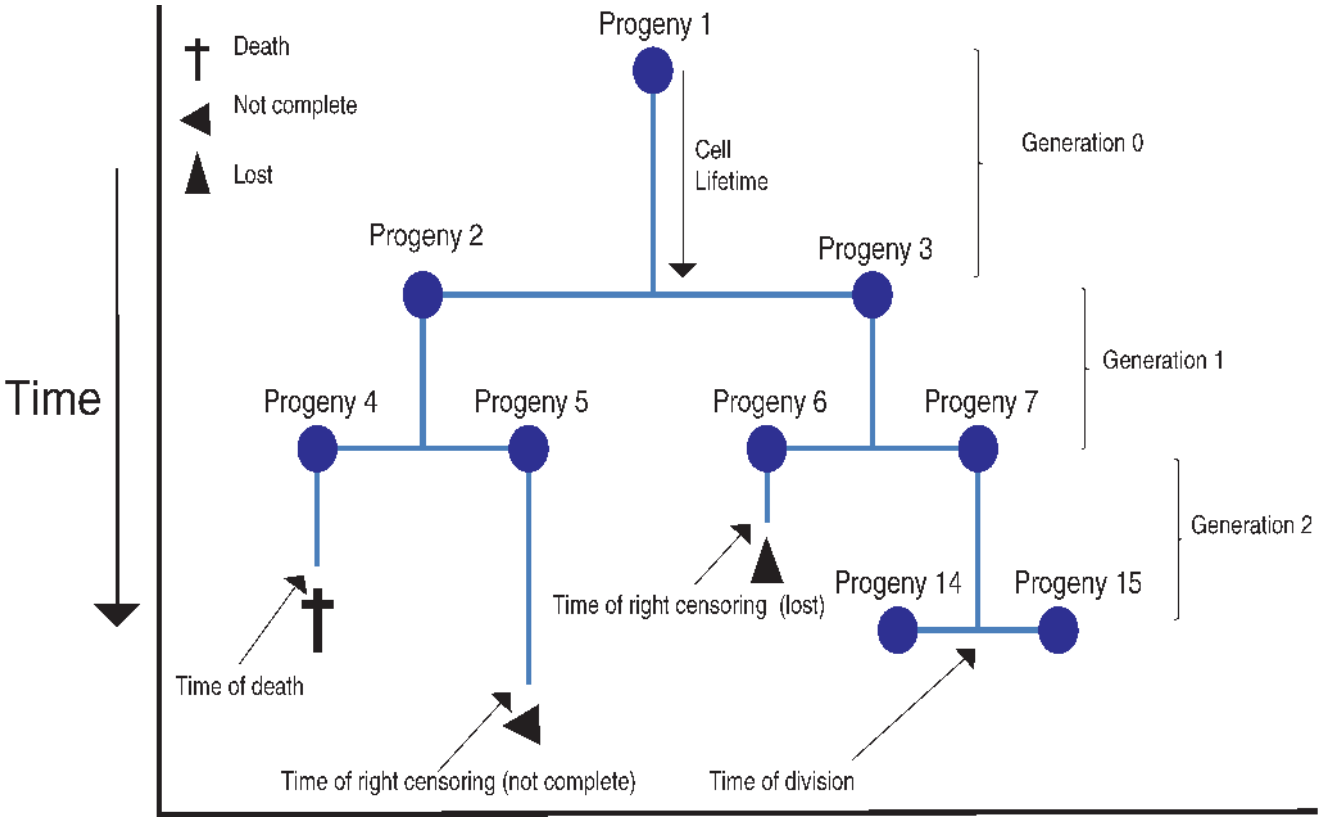



**Figure 24.5** An example of single cell pedigree annotation. Times of cell fate outcomes (death, division, lost, not complete) are recorded for all tracked cells. Cells within a pedigree are referred to as progeny and are numbered according to their position within the pedigree. Any single cell measurement, such as size or reporter expression, can be annotated.

**Box 24.1** Web-based resources for live cell imaging and cell tracking.

**Live cell imaging systems**
- http://www.zeiss.com/microscopy/en_de/solutions/bioscience-tasks-applications/live-cell-imaging.html
- http://olympusmicro.com/index.html
- http://www.leica-microsystems.com/sciencelab/topics/live-cell-imaging/

**Cell tracking platforms**
- http://www.cellprofiler.org/ (CellProfiler)
- https://github.com/NICTA/TrackAssist/wiki (TrackAssist)
- http://www.cellcognition.org/ (CellCognition)
- http://fiji.sc/TrackMate (Fiji plug-in)
- http://rsb.info.nih.gov/ij/plugins/track/track.html (ImageJ plug-in)

**Image analysis software**
- http://imagej.nih.gov/ij/ (ImageJ)
- http://fiji.sc/Fiji (Fiji)
- http://au.mathworks.com/products/image/ (Matlab)
- http://www.moleculardevices.com/systems/metamorphresearch-imaging/metamorph-microscopy-automation-andimage-analysis-software (MetaMorph)

**Useful microscopy references**
- http://www.microscopyu.com/articles/fluorescence/
- http://www.leica-microsystems.com/applications/lifescience/fluorescence/

dendritic cell and MSC motility, an effect that was strengthened by the addition of TNF-α and reversed by the anti-inflammatory factor resveratrol. Disadvantages of these platforms are that they do not necessarily include tools for quantifying reporter signals, annotating fate outcomes and morphological features, or extracting kinship relationships.

Establishment of kinship relationships is uniquely achievable by single cell tracking and is an important feature of pedigree data. Kinship relationships can only be established by following every cell continuously from birth until division and annotating mothers and their progeny (Figure 24.5). However, this functionality could be added to open-source cell tracking platforms through modification to existing code. Alternatively, there are academic cell tracking platforms that include both basic and advanced cell tracking features. Examples with proven utility include Timm's Tracking Tool (TTT) [20], developed by the Stromal Cell Dynamics group at the Swiss Federal Institute of Technology; TrackAssist, developed between Australia's National Information and Communications Technology Association and researchers at the Walter and Eliza Hall Institute [21]; Nordon's Tracking Tool (NTT), developed at the Graduate School of Biomedical Engineering, University of New South Wales (R.M. Hallett *et al.*, University of New South Wales,

unpublished results); and CellProfiler, developed at The Whitehead Institute for Biomedical Research and The Massachusetts Institute of Technology (Box 24.1).

Cell tracking platforms are classified as automated or manual tracking platforms. CellProfiler is an example of an open-source automated image analysis and cell tracking platform implemented in Matlab and Python [40]. Initially, it was developed as a tool for biologists to identify and quantify cell phenotypes [40,41]. Automated cell tracking platforms such as CellProfiler require image segmentation to define cells as objects in each frame and algorithms to identify each object's trajectory in consecutive frames in order to build tracks. Once tracks are built for a particular object then measurements related to that object can be annotated to its track. Automated cell tracking platforms have the capacity to dramatically increase throughput, although their utility is often restricted. This is because image segmentation and tracking algorithms are not suitable for cell cultures that are highly proliferative or confluent, since individual cells are difficult to recognize even by eye. In addition, tracking algorithms reliant on accurate segmentation rely on images that are easily segmented (e.g., fluorescently labeled nuclei and phase-contrast images of round cells). Thus, automated tracking is not suitable for all cell types and can produce errors that require manual correction, a process that is as time consuming as manually tracking cells [6]. Automated tracking software packages have implemented algorithms to detect events of interest, such as cell division, death, and differentiation [42,43]. On the other hand, manual cell tracking platforms, such as TTT and NTT, require analysts to identify the cell in each frame and annotate its fate outcomes manually while tracking. This requires continuous observation from the user and thus is more labor intensive than automated cell tracking platforms. Importantly, manual tracking allows for errors to be corrected in real time. NTT is a custom-written cell tracking platform implemented in Matlab. It was developed as a flexible tracking platform for tracking cells from a range of different cellular systems in either transmitted light or fluorescent microscopy images (see Section 24.4.2.5. for a more detailed description of NTT).

Basic features, such as cell identification labels, positional information, morphological parameters, and reporter signals, are recorded by most cell tracking platforms. The default cell tracking parameters provided by CellProfiler are shown in Table 24.1. The default cell tracking results from NTT are provided in Table 24.2 for comparison. These platforms are both open source, so functionality can be added by any user with Matlab programming skills. Therefore, single cell tracking platforms such as these can be adapted to overlay any cellular measurement onto single cell pedigrees.

**Table 24.1** Cell features recorded by default by CellProfiler cell tracking platform.

| Measurement or feature | Description |
|---|---|
| TrajectoryX | Distance traveled in *X* direction between consecutive frames |
| TrajectoryY | Distance traveled in *Y* direction between consecutive frames |
| Label | A unique identifier for the tracked object. When objects merge or split new labels are created. |
| ParentImageNumber, ParentObjectNumber | The ImageNumber and ObjectNumber of the parent object in the prior frame. For a split, each child object will have the label of the object it split from. For a merge, the child will have the label of the closest parent. |
| DistanceTraveled | Euclidean distance traveled by the object from the previous frame to the current frame. |
| Displacement | Distance traveled by the object from its initial position in the first frame to the position in the current frame. |
| IntegratedDistance | Total distance traveled by the object between the first and last frame. |
| Linearity | A measure of how linear the object trajectory is during the object lifetime. Calculated from the ratio of the displacement and integrated distance. |
| LifeTime | The number of frames an object has been tracked through. Lifetime starts at 1 in the frame when an object appears, and is incremented with each frame that the object persists. |
| FinalAge | Similar to LifeTime but is only output at the final frame of the object's life (or the movie ends, whichever comes first). At this point, the final age of the object is output; no values are stored for earlier frames. Useful for generating histograms of object lifetimes. |

### 24.3.2 Recording live cell characteristics

Live cell imaging experiments utilizing transmitted light microscopy provide enough information to generate cell trajectories, though fluorescent reporter labeling of the nucleus will significantly shorten tracking times. Cell migration is quantified from cellular (*X*, *Y*, *T*) coordinates, which are used to calculate displacement, total distance, directionality, and instantaneous and average velocities under different conditions. Such analyses have been applied to studying how physiologically relevant direct current electric fields of the order of 10–200 mV/mm, such as those established at sites of bone fracture, are able to stimulate MSC-directed migration [15]. In addition, the position of MSCs relative to each other and their microenvironment can be recorded and related to their growth dynamics; for example, a cell's location with respect to the center or edge of a colony, or whether a cell's duration of contact with neighboring cells alters cell fate [12]. The shape of the cell at each time point can be measured to provide morphological parameters such as area, length, eccentricity, and nuclear to cytoplasm ratio. Importantly,

**Table 24.2** Cell features recorded by default by NTT cell tracking platform.

| Measurement or feature | Description |
|---|---|
| Clone ID | A unique identifier for each clone. Starts at 1 and when a new clone is tracked. |
| Progeny ID | A unique identifier for cells within a clone. Founding cell starts at 1 and cells are numbered depending on their position within the pedigree structure. |
| TrackNumber | A unique identifier that corresponds to the order in which cells are tracked. Starts at 1 for the first track. |
| X and Y coordinates | The *X* and *Y* pixel coordinates for the position of the cell. 0, 0 is the top left corner. |
| Timestamp | A time vector containing the timestamps from the consecutive images in which cells are tracked. |
| BirthTime | A date vector containing the time the track started. For example, birth or left censoring. |
| DeathTime | A date vector containing the time the cell track finished. For example, death, division, lost cell, experiment finished. |
| LifeTime | A date vector containing the time elapsed between the BirthTime and DeathTime. |
| StopReason | A code for the reason a cell track was stopped:<br>0 – Fate not observed before final frame<br>1 – Division<br>2 – Death<br>3 – Lost cell<br>The time the track is stopped corresponds to the DeathTime. |
| CellMask | A matrix containing indices for each pixel in the tracking ellipse. Tracking ellipse is an adjustable ellipse placed over the nucleus of the cell for tracking. |
| Endomitosis | A binary variable and timestamp indicating if a cell has endoreduplicated and the time it occurred. 0 is default and 1 indicates endomitosis. |
| Reporter signal quantification (Max, Mean, Interpolated) | A vector with measurements from fluorescent channels. For example, for fluorescent reporters. |

the change in shape of a cell can be monitored over time and observed in response to external stimuli such as pharmacological and toxicological agents or to perturbations of signaling pathways [44]. While such observations are insightful, fundamental information, such as the age of a cell when it dies or divides, allows one to characterize and model cellular proliferation and cell fate choices at a level of detail that well exceeds standard methods, such as flow cytometry and clonogenic assays.

MSC division is easily identified from transmitted light images as MSCs become semi-adherent at the beginning of mitosis. Cells then split into two semi-adherent cells and completely separate at the end of cytokinesis before re-adhering as two separate cells. MSC death is identified when cells stop moving before membrane degradation. One is unable to distinguish apoptosis (programmed cell death) from necrosis without fluorescent markers identifying apoptosis (e.g., conjugated fluorescent monoclonal antibodies to Annexin V).

Morphological and behavioral characteristics of MSCs can also be related to their fate outcomes to identify how proliferation and differentiation generates heterogeneity [12]. Figure 24.6A shows a single cell pedigree where both twins divide in the first generation, yet in the second generation there are unequal generation times and cell age at division appears to correlate with cell area. Further quantification of extrinsic factors (cell–cell contact) and intrinsic factors (cell division history and cell area) showed that extrinsic and not intrinsic factors influenced division heterogeneity (Figure 24.6B and C). Furthermore, β-galactosidase staining revealed that the majority of large cells were senescent (Figure 24.6D).

Another feature that can be extracted from time-lapse imaging and cell tracking experiments is the inherited relationships between MSCs derived in culture. A single cell pedigree depicts the kinship relationships between members of the same clone, namely siblings, mothers and daughters, cousins, and so on. Therefore, kinship relationships can be used to study how MSC growth dynamics varies between nonrelated and related cells and how cell traits are transmitted through successive generations [45]. Such analyses have been used to study how latent heritable factors can influence cell growth dynamics as well as studying MSC heterogeneity [46,47]. There is a wealth of information that can be obtained from recording the dynamics of MSC migration, morphology, kinship relationships, and life history. However, cell fate biomarkers are required to dissect the molecular control of cell fate.

### 24.3.3 Vital biomarkers for mesenchymal stromal cells

Morphological and behavioral properties of MSCs are easily recorded using conventional phase-contrast, bright-field, or DIC microscopy in conjunction with appropriate cell tracking and image analysis software. To supplement this information and to answer more specific questions, vital biomarkers are required to relate molecular processes to cell development. Vital biomarkers can include fluorescent stains that mark specific cellular compartments or functional processes, genetic labeling with fluorescent protein probes, as well as novel fluorescent particles such as QDs [48] or nanoparticles (NPs) [13].

Differential labeling by vital stains provides additional information to help distinguish distinct cell types within a heterogeneous population (i.e., stem cells, progenitor cells, or differentiated cells) [13]. Importantly, labeling information can be overlaid on to single cell pedigrees, allowing one to correlate gene expression, cell ultrastructure, or metabolic processes with cell age, cell fates, or phenotype (e.g., morphology or

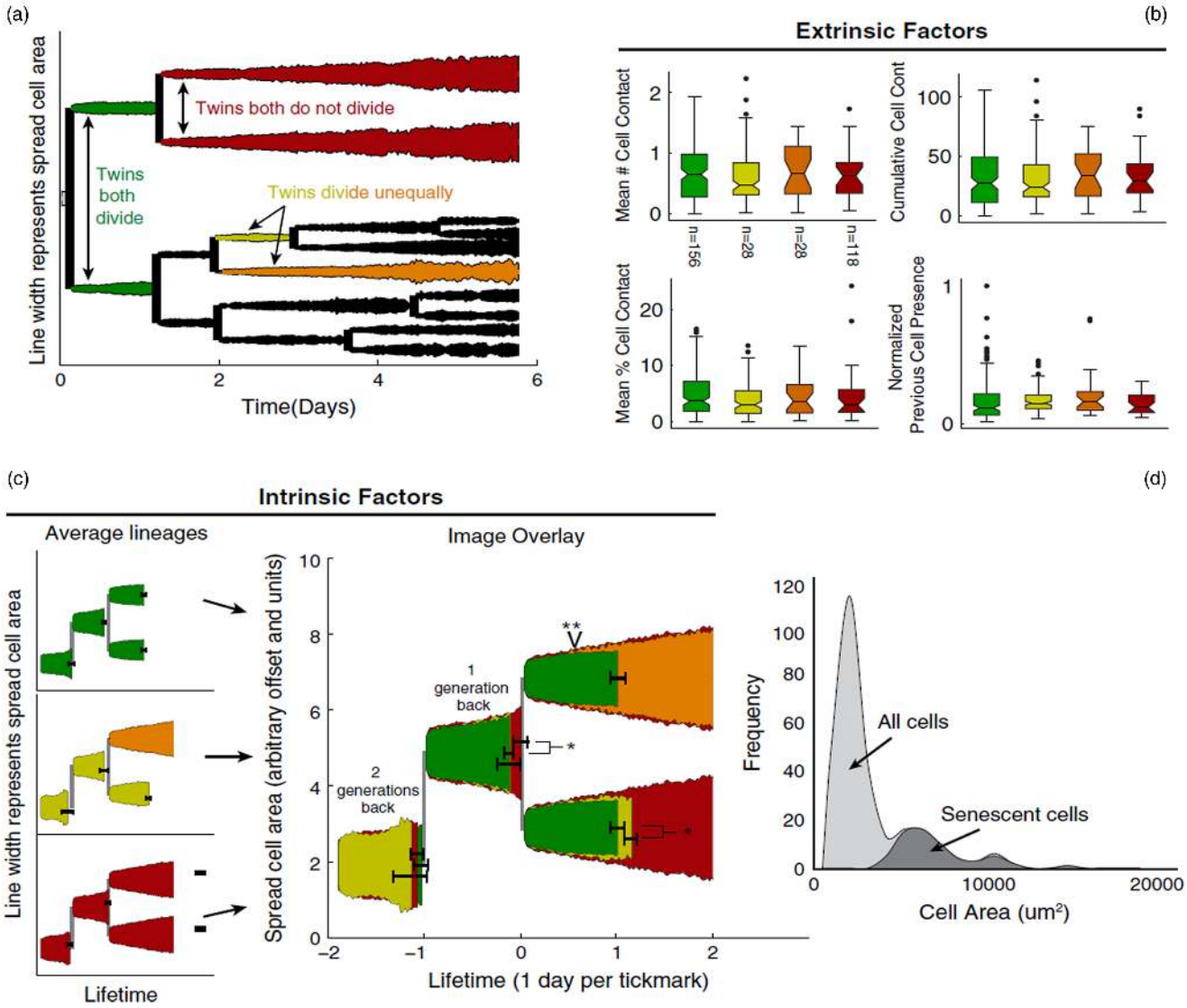



**Figure 24.6** Potential sources of size heterogeneity among MSCs. (A) A cell pedigree with heterogeneous divisions. Pedigree contains siblings that both divide, one divides and one does not, or both do not divide. (B) Extrinsic factors that could potentially influence time to cell division. Contact was defined as another cell within 12.9 μm proximity. No significant differences were observed for extrinsic factors. (C) The average lineage history of the cells (intrinsic factors) for each of the three groups overlaid and individually (inset). Line width represents spread cell area; 95% confidence intervals for lifetime are represented by error bars. ∗ denotes significance at the $p < 0.05$ level; ∗∗ marks the time that the cell area for dividing and nondividing cells becomes significantly different ($p < 0.05$). (D) Histogram showing distribution of cell sizes, including those stained positive for the cell senescence marker β-galactosidase after 7 days' culture. *Source:* [12] Whitfield *et al.*, 2013, pp. 1365–1377. Reproduced with permission of Elsevier.

motility). Thus, fluorescent labeling is crucial to gaining a molecular understanding of cellular dynamics.

The most commonly applied methods for labeling MSCs are fluorescent dyes and genetically encoded fluorescent proteins. When selecting a fluorescent label the spectral properties of the fluorophore must first be considered. These properties include emission and excitation wavelength, extinction coefficient, quantum efficiency, brightness, and photobleaching. Excitation wavelength is the wavelength of a photon that is required to excite the fluorophore. When a fluorophore is excited by an incident photon it will emit a photon with a longer wavelength than the photon it had absorbed. The wavelengths of the incident and emitted photons are referred to as the excitation and emission wavelengths respectively. Light sources, filters, and cameras capable of producing and detecting light at the excitation and emission wavelengths are necessary. Brightness is another important property of any fluorophore being used in a labeling strategy. Brightness of a fluorophore is determined by its extinction coefficient, its quantum efficiency, and its susceptibility to photobleaching [49]. The extinction coefficient is a measure of

how much of the excitation light is absorbed in relation to the concentration of the fluorophore in solution; that is, it is the efficiency of a fluorophore at absorbing photons at the excitation wavelength. Quantum efficiency is a ratio of the number of photons emitted to the number of photons absorbed. Photobleaching refers to a fluorophore that has been rendered into a nonfluorescent state due to the absorption of photons.

In addition to the properties of the fluorophore itself one must consider that detection of the fluorophore is dependent on the signal-to-noise ratio. The signal-to-noise ratio is not only dependent on the fluorophore's brightness but also on the level of background noise and autofluorescence from cells, medium, and tissue culture vessels. In order to improve signal-to-noise ratio one should attempt to reduce the degree of background noise and autofluorescence. This can be achieved by selecting appropriate medium; for example, a medium without phenol red and a tissue culture substrate that has low autofluorescence. Tissue culture vessels manufactured with a thin-film substrate are ideal since the reduced thickness significantly reduces background fluorescence. Multiwell plate designs with black walls are also suitable as they prevent internal reflections that introduce additional light into the FOV.

Cell tracking dyes such as carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) can be used to track cell division because with physically symmetric divisions the fluorescence intensity halves with each successive mitosis [50]. When CFDA-SE-stained hematopoietic cells are initially sorted and then analyzed by flow cytometry, successive cell generations are identified on CFDA-SE histograms as peaks whose mode decreases geometrically by a factor of 2 [51]. Differentiation is tracked with respect to generation number using multicolor flow cytometry [52]. Cell division tracking dyes are diluted to an undetectable level after several rounds of cell division [53]. Proliferation, survival, and renewal can be calculated by counting the number of cells belonging to successive generations [50,54,55]. However, CFDA-SE division tracking has not been applied to MSCs because cell division is not always physically symmetrical.

Fluorescent labeling of cell nuclei greatly assists with MSC tracking because the nucleus of the cell is easily identified by threshold segmentation (Figure 24.3B). DNA-staining agents such as Hoechst 33342 and DAPI intercalate with DNA and are genotoxic. A better approach is to label nuclei genetically with a fluorescent protein.

There are also fluorochromes that quantify various aspects of cellular metabolism. For example, CFDA-SE and calcein acetoxymethyl ester become fluorescent upon hydrolysis by intracellular esterases [48]. Acetylated (Ac)-low-density lipoprotein (LDL) conjugated to a fluorophore can be used as a functional assay to identify ECs and macrophages [56].

The cloning of the GFP from *Aequorea victoria* revolutionized the field of live cell imaging by providing a palate of fluorescent proteins for reporting gene expression in transgenic mouse models and transduced or transfected primary cells and cell lines. Colored variants of GFP, such as red fluorescent protein (RFP), yellow fluorescent protein (YFP), and cyan fluorescent protein (CFP), can be detected independently with the potential to quantify several genes simultaneously. Fluorescent proteins that have been expressed in MSCs without any toxic or adverse effects on cell function include enhanced GFP (eGFP) [23], DsRED [57], enhanced CFP [58,59], YFP [58], Venus [60], and RFP [59].

A fundamental limitation of using fluorescent proteins for labeling cells is that an overlap in excitation and/or emission spectra is inevitable as more labels are introduced. Even though excitation and emission channels can be imaged in sequence, it may not be possible to compensate for spectral overlap. Spectral mixture analysis can deconvolve emission spectra using multiple images [30], though increased sample illumination, photobleaching, and phototoxicity is the trade-off.

Fluorescent proteins modified with amino acid sequences or fused to larger ligands can localize fluorescence to various subcellular sites. For example, a fluorescent protein fused to histone H2B will label the nucleus. The cytoskeleton is labeled using α-tubulin as a fusion partner. The inner leaf of cell membranes is labeled using a basic membrane localizing sequence isolated from the Ras oncoprotein [61]. Nuclear localizing sequences can be used to transport fluorescent proteins into the nucleus. The spatial distribution of these reporters is distinct, so spectral unmixing is not required [4].

QDs and NPs have specific advantages for labeling and tracking MSCs. QDs are NPs made from semiconductor nanocrystals. QDs are bright with a high extinction coefficient and similar quantum yield to fluorescent proteins and are photostable with less photobleaching than fluorescent proteins [62,63]. QDs have been shown to have no significant adverse effects on functional characteristics of MSCs, such as proliferation and differentiation capacity *in vitro* [63], though there have been issues regarding inconsistent retention within MSCs [64,65] and increased apoptosis due to toxicity at high concentrations [48,62]. QDs have even been used for labeling MSCs based upon specific integrin expression; for example, the immobilization of the peptide CGGGRGD on CdSe–ZnS QDs [63], as well as for identification of labeled MSCs in cardiomyocyte co-culture assays [64].

Fluorescent NPs are fabricated using organic matrices or polymers that can be formed into a variety of shapes, enabling intracellular measurements in living cells [66]. Nanoflares are an example of NPs that are used to visualize and quantify relative levels of mRNA transcripts within living cells. Nanoflares are comprised of spherical nucleic acids conjugated to gold nanoparticles. The gold nanoparticles are densely packed with oligonucleotide sequences that correspond to their mRNA target. The oligonucleotide sequences are hybridized to fluorophore labels that fluoresce when the oligonucleotide is exposed to its mRNA target. Multiplexed nanoflares have also been developed that allow

for the simultaneous detection of two distinct mRNA targets within a living cell [67].

Viable biomarkers are crucial tools for live cell imaging as they allow dynamic molecular processes to be visualized and measured. Single cell biomarker signal measurements can be overlaid onto single cell pedigrees to enable a cell's molecular or functional state to be related to its life history.

### 24.3.4 Mimicking *in vivo* microenvironments in vitro

A major drawback and a frequent criticism of studying stromal cells *in vitro* is that when they are removed from their *in vivo* niches their properties are immediately altered. Thus, cellular responses observed *in vitro*, particularly those related to differentiation potential, may be tissue culture artefacts that are not physiologically relevant [68–70]. However, the clinical potency of MSCs is currently defined in part by *in vitro* assays and immunophenotype. The International Society for Cell Therapy (ISCT) stipulates that a functional property of MSCs is the ability to adhere to tissue culture plastic and to proliferate long term *in vitro*.

While *in vitro* microenvironments do not simultaneously provide all physiological biomechanical and biochemical stimuli, their utility is related to a more precise analysis of the effect of specific stimuli. Various features of the *in vivo* niche can be recapitulated by engineering well-controlled artificial *in vitro* microenvironments [71].

The *in vivo* MSC niche is defined as the immediate microenvironment surrounding an MSC and has a nanometer to a micrometer length scale. Its microenvironment is comprised of biomechanical and biochemical cues that vary temporally and spatially to regulate cell survival, activation, self-renewal, and differentiation [71]. These cues include, but are not limited to, autocrine and paracrine growth factor stimulation, cell–cell contacts, and cell–matrix interactions (Figure 24.7).

Examples of bioengineered niches for MSCs include functionalized surfaces [72], modular biomaterials [73], three-dimensional scaffolds [74], and microfluidic devices that can generate physiologically relevant soluble and fixed gradients [75,76]. More sophisticated microfluidic systems have been designed to simulate the cardiac cycle by generating pulsatile shear flow [77]. Such *in vitro* microenvironments are suitable for dissecting the role of biomechanical and biochemical stimuli on MSC fate. For example, the effect of substrate stiffness on osteogenic differentiation of MSCs was studied by culturing cells in polyethylene glycol (PEG) hydrogel microwells with variable stiffness (shear moduli of 1–50 kPa) [78]. Using this system in conjunction with live cell imaging, an increase in substrate stiffness resulted in a significant increase in osteogenic differentiation. Substrate stiffness is well known to be a biomechanical stimulus that affects both MSC lineage specification and self-renewal ability [73,79,80].

In order to probe for effects of ECM interactions on stromal cell fate one is able to culture cells on substrates functionalized with micropatterned ECM proteins by microstamping [81] or robotic spotting [78]. Cell–cell interactions can also be mimicked *in vitro* by solid-phase immobilization of biomolecules such as cadherin. These solid-phase systems have been successfully employed to study the effect of N-cadherin and cell-adhesive fibronectin fragment 9–10 (FN9–10) on the adipogenic differentiation potential of MSCs (Figure 24.8). FN9–10 and

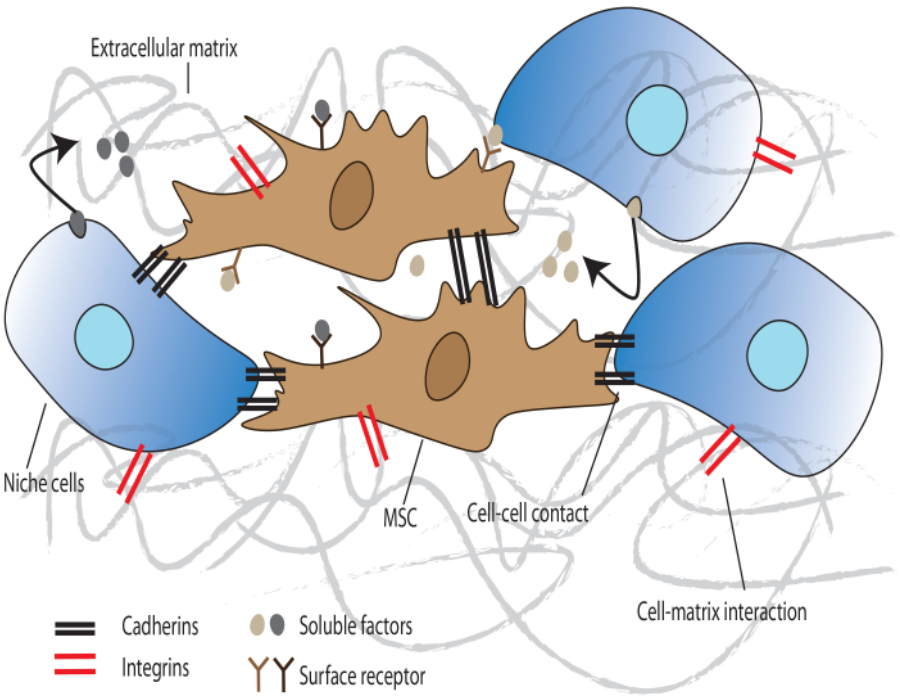



**Figure 24.7** Stylized cartoon of the *in vivo* MSC niche showing cell–cell interactions, cell–matrix interactions, soluble factor production, and stimulation of MSCs by supportive niche cells. The extracellular matrix (ECM) provides biochemical stimulation of MSCs through integrin interactions and tethered factors as well as mechanical stimulation of MSCs by its viscoelastic properties.

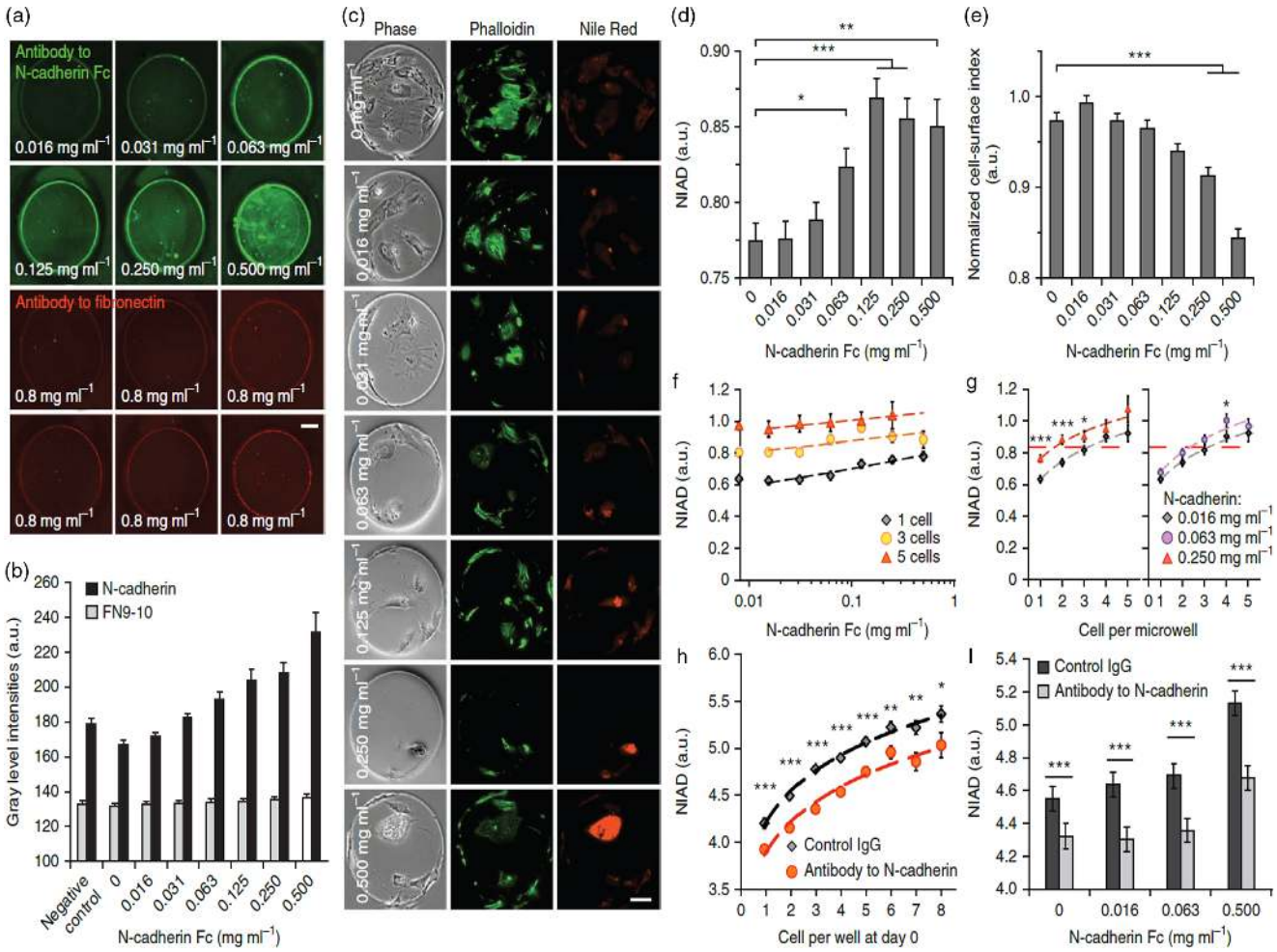



**Figure 24.8** Cell density effect on MSC adipogenic differentiation. (a) Immunostaining of microwells functionalized both with N-cadherin and with N-cadherin and FN9–10. (b) Plot showing quantification of the signal after immunostaining microwells containing N-cadherin and FN9–10. Negative control: PEG. (c) Phase contrast (Phase), phalloidin–Alexa Fluor 488 (Phalloidin), and Nile red images of MSCs exposed to varying concentrations of N-cadherin. All scale bars 100 μm. (d, e) Adipogenic differentiation (d) and cell-surface index (e) plotted against N-cadherin concentrations. NIAD: normalized index of adipogenic differentiation. (f) Adipogenic differentiation as a function of N-cadherin concentration for microwells with indicated number of initial cells. (g) Dependence of adipogenic differentiation on cell density at varying N-cadherin concentrations. The red dashed line represents the average adipogenic differentiation across all microwells. (h, i) Effect of blocking N-cadherin on adipogenic differentiation for varying initial cell densities (h) and N-cadherin concentrations (i). IgG: immunoglobulin. Error bars, standard error of the mean as computed after a multivariate analysis (generalized linear model) on $n = 5167$ (gain of function experiments) and $n = 5860$ (loss of function experiments) observations. $***p < 0.001$, $**p < 0.01$, and $*p < 0.05$; a.u.: arbitrary units. (See color plate section.) *Source:* [78] Gobaa 2011, pp. 949–957. Reproduced with permission of Nature Publishing Group.

N-cadherin were functionalized on microwells at different densities (Figure 24.8a and b). After 11 days in culture, actin network and intracellular lipid accumulation were visualized by phase contrast, fluorescently labeled phalloidin, and lipid staining with Nile red (Figure 24.8c). N-cadherin exposure significantly increased adipogenic differentiation and decreased the surface area of MSCs in a concentration-dependent manner (Figure 24.8d–g). Furthermore, a blocking antibody to N-cadherin significantly reduced adipogenic differentiation potential (Figure 24.8h and i). It is interesting to note that the average cell surface area was also significantly reduced with increasing N-cadherin concentration, revealing how cytoskeletal architecture is influenced by cell–cell interactions. In more elaborate systems, different protein solutions can be printed onto microwells for factorial analysis of the effect of these proteins on stromal cell fate outcomes. Such a system has been applied to studying neural stromal cell fate outcomes in the presence of the NOTCH ligands JAGGED and Delta like ligand 4 [78].

Mimicking of various signals that comprise the *in vivo* microenvironment of MSCs will provide researchers with greater power to understand their *in vivo* roles. The advantage of *in vitro* systems is that cell fate is more accurately quantified by live cell imaging. Indeed, if meaningful microenvironments

are created, then live cell imaging and single cell tracking experiments can provide more relevant insights into MSC biology [71].

## 24.4 Case study: tracking differentiation of endothelial cells from cardiac-derived mesenchymal stromal cells

### 24.4.1 Background and experimental aims

The aforementioned general principles of live cell imaging will now be applied to an analysis of MSC differentiation. Most of the art in this field relates to software analysis of time-lapse movies. Here, we present a detailed case study illustrating the tracking of cardiac-derived MSC-to-EC transition. ECs are cells that line the luminal surface of all blood vessels and are essential to the homeostasis and repair of organ compartments [82]. There is great interest in generating various EC populations, including progenitors for regenerative medicine applications; for example, in cardiovascular regeneration, as new tissue requires a blood supply and a vascular network to define its growth and architecture [83]. The limited expansion capacity and lack of autologous sources for ECs has hampered their use in therapy [84]. There is increasing interest in the potential of MSCs to be differentiated into ECs for clinical applications. Several important factors required to induce endothelial differentiation for MSCs have been identified. These factors include endothelial growth supplements, shear force, and ECM components. Several reports detail EC marker expression and maturation derived from adipose-tissue-derived MSCs [85,86] or bone-marrow-derived-MSCs [83,87–89].

Cardiac colony-forming units–fibroblast (cCFU-Fs) are a multipotent, cardiac-resident MSC-like population. cCFU-Fs are hypothesized to be involved in tissue remodeling of the heart during homeostasis and in response to injury, for example, in response to myocardial infarction. Relevant to their hypothesized role in cardiac tissue remodeling, cCFU-Fs have the potential to differentiate into an EC phenotype *in vitro* when stimulated with vascular endothelial growth factor (VEGF) for 12–14 days [23]. Uptake of fluorescently labeled Ac-LDL, a functional assay for the identification of ECs, was used to track endothelial differentiation of cCFU-Fs. After differentiation, cells were exposed to 10 μg/mL of fluorescently labeled Ac-LDL for 24 h, with 60–80% of cells showing significant accumulation of Ac-LDL [23].

Ac-LDL is a major carrier of cholesterol in the blood, and uptake of LDL occurs in a number of cell types, including macrophages and ECs [90]. Ac-LDL uptake occurs through LDL-receptor-mediated endocytosis during which LDL is internalized and degraded [91]. The Ac-LDL uptake assay has been widely used as a functional assay for the identification of ECs [90]. From a live cell imaging perspective, Ac-LDL is a useful vital biomarker that can be used to identify phenotype transitions, such as from hemogeneic endothelium to a hematopoietic phenotype [17].

Immunocytochemical staining and flow cytometry analyses are also routinely used to identify cells that express markers of the endothelial lineage, such as von Willebrand factor and VE-cadherin [23]. These assays quantify efficiency of differentiation by simply providing counts of marker-positive cells before and after differentiation [92]. The potency of an MSC population can be easily established by this method. However, these methods do not provide insight into the dynamics of lineage commitment. In fact, such assays only reveal that a proportion of the initial cell population has survived and differentiated into ECs.

The time course of immunocytochemical, fluorescence-activated cell sorting (FACS), and gene expression analyses will reveal differentiation dynamics of MSC populations. However, lineage commitment occurs at the single cell level, so it is more appropriate to study differentiation by continuously tracking single cells. For example, one can evaluate instructive versus permissive hypotheses for lineage development in the MSC system. The instructive hypothesis implies that cCFU-Fs will be directed to change their phenotype in response to endothelial growth conditions, whereas the permissive hypothesis states that these conditions promote survival and selection of MSCs that are EC progenitor cells. Furthermore, continuous observations of single cells allow one to determine how cCFU-F lineage commitment can influence the probability of other cell fate outcomes, such as division and death.

This case study will demonstrate the application of live cell imaging and single cell tracking to MSC differentiation. Endothelial differentiation was tracked using uptake of Ac-LDL. We addressed the following:

1 Tracking of cCFU-Fs in serum-free medium (SFM) or with endothelial growth medium (EGM).
2 Recording the dynamics of fluoroscein isothiocyanate (FITC)–Ac-LDL uptake to identify phenotype transitions.
3 Analysis of the following fate outcomes:
   **(a)** phenotype transition
   **(b)** division
   **(c)** death.

### 24.4.2 Methods

#### 24.4.2.1 Cardiac colony-forming unit isolation and culture

cCFUs were isolated from 8- to 12-week old wild-type C57BL/6 mice. Mice were sacrificed by cervical dislocation, hearts removed, and the interstitial cell fraction isolated by mechanical and enzymatic digestion with collagenase type II (Worthington) at 37 °C for a total of 30 min. Every 10 min the sample was triturated mechanically with a transfer pipette to ensure complete digestion. Supernatant was collected and filtered through a 40 μm strainer (to remove cardiomyocyte debris) into 1% fetal calf serum to stop digestion. Red blood cells were lysed with a red cell lysis buffer followed by dead cell removal using MACS Dead Cell Removal Kit (Milteny Biotec). Cells were then incubated for 30 min at 4 °C with

fluorophore-conjugated antibodies against stem cell antigen (Sca-1), platelet-derived growth factor receptor-α (PDGFR-α), and platelet EC adhesion molecule (PECAM, CD31). Using either FACS Aria or FACS Canto (Becton, Dickinson) cytometers, cCFU-Fs were enriched as Sca-1$^+$/PDGFR-α$^+$/PECAM$^-$. Cells were collected in a 15 mL Falcon tube with 2 mL serum-free medium containing 2 ng/mL of basic fibroblast growth factor (bFGF) and 1 ng/mL of transforming growth factor receptor-β1. After isolation, the enriched fraction of cCFU-Fs was centrifuged at 300*g* for 5 min, the supernatant removed, cells resuspended in 200 μL and then counted manually using a hemocytometer. Twenty-four hours after initial isolation the medium was removed and cells were washed twice with phosphate-buffered saline to remove nonadherent cells. For *in vitro* expansion, cells were plated at 150 cells/cm$^2$ in 10 cm petri dishes (BD Falcon) and cultured in SFM for 12 days with medium changes every 3–4 days. All tissue culture vessels were coated with attachment factor (Invitrogen) for a minimum of 0.5 h at 37 °C before plating cells. Attachment factors are structural proteins or protein-like substances that have adherent capabilities and increase cell–substrate interactions in a culture-dependent attachment milieu. A number of glycoproteins have been identified that promote and/or influence *in vitro* cell attachment to the surface or substratum of the culture vessel, and these include gelatin, ECM, fibronectin, and collagen type-1.

#### 24.4.2.2 Endothelial differentiation and fluorescein isothiocyanate–acetylated low-density lipoprotein uptake

For EC differentiation, cCFU-Fs expanded in SFM to passage 4 were used. cCFU-F cultures were plated at 250 cells/cm$^2$ in 24-well special-optics live cell imaging plates (Ibidi, Germany) in either SFM or EGM. EGM consisted of 1% fetal calf serum (Gibco, Australia) in Iscove's modified Dulbecco's medium (IMDM) (Invitrogen, Australia) containing 10 ng/mL bFGF (R&D, USA) and 10 ng/mL VEGF (R&D, USA). To induce differentiation into an endothelial phenotype, cells were cultured for 12 days. Medium was replaced every 3–4 days. In order to visualize cCFU-F to endothelial transitions in real time, 10 ng/mL FITC-conjugated Ac-LDL (Invitrogen) was added directly to both control (Dulbecco's modified Eagle medium (DMEM)) and differentiation conditions at 10 ng/mL. Fresh Ac-LDL was added each time medium was replaced.

#### 24.4.2.3 Live cell imaging

Once cells had attached, the plate was placed on a Leica inverted live cell imaging microscope (DMI6000B, Leica, Australia) equipped with a fully-automated *X*–*Y*–*Z* controller and hardware autofocus control. The microscope was housed in a large closed-chamber incubator box (PeCon, Germany). The sample environment was maintained at 37 °C using a heating unit and temperature controller (PeCon, Germany). A $CO_2$ controller (PeCon, Germany) was used to deliver a 5% $CO_2$ and air mixture through a small-volume cover placed over the live cell imaging plate. Default functions contained within the MetaMorph Premiere Software package (MM, MolecularDevices, USA) were used to define image acquisition parameters and control the microscope. Nine positions in the center of each well (forming a 3 × 3 contiguous grid with an overlap of 0.5 μm) of the 24-well plate were selected for imaging. Two wells for each condition were imaged. Phase-contrast images at 20× were acquired every 20 min with an exposure time of 1 ms. Phase-contrast images were acquired for cell tracking and to visualize morphological changes of cCFU-F cultures from a mesenchymal (spindle shaped) to an endothelial morphology (cobblestone appearance). To minimize phototoxicity and photobleaching effects, Ac-LDL fluorescent images were acquired every 2 h with a 750 ms exposure time. An acquisition frequency of 2 h was sufficient to capture Ac-LDL accumulation within individual cells yet did not cause phototoxicity. Every 3–4 days dishes were removed from the microscope and the medium was changed, including addition of FITC–Ac-LDL. Medium changes were performed in less than 20 min (less than the acquisition frequency). When returning the imaging plate back to the microscope stage, the last acquired imaged was used as a reference to ensure that the same FOVs were imaged.

#### 24.4.2.4 Data storage and image processing

All images were saved as .TIF files (1392 × 1040 resolution, 2.5 MB file size) on the local disk of the same computer running MetaMorph and controlling the microscope. At the end of the experiment, copies of all raw images were transferred to an external hard disk (4 TB, Western Digital) for long-term storage. Custom-written scripts implemented in Matlab were used for stitching contiguous FOVs, background removal, flattening of uneven illumination, and contrast enhancement. Flattening of uneven illumination in fluorescent images was most important as the FITC–Ac-LDL intensity was quantified by measuring pixel intensities after background subtraction.

#### 24.4.2.5 Cell tracking

All cell tracking was performed using NTT implemented on a graphics workstation equipped with a graphical processing unit (HPZ420, 16 GB RAM, Intel® Xeon® CPU E5-1607 @ 3.00 GHz). NTT reduces the labor of cell tracking using a template-matching algorithm that recognizes the cell in the current frame using past images of the cell as a learning set. The program automatically tracks the cell's position, but will revert to manual tracking if the cell image recognized by the algorithm is outside statistical confidence limits. It is best to track a cellular structure that is invariant during tracking, such as the cell nucleus. The graphical interface consists of a software controller and movie viewer (Figures 24.9 and 24.10). Cells were tracked in phase-contrast images by placing an ROI over the cell nucleus and double-clicking. The ROI is an adjustable ellipse (default 35-pixel diameter) used to record the position of the nucleus as well as all pixels contained within. The pattern of pixels recorded within the ROI

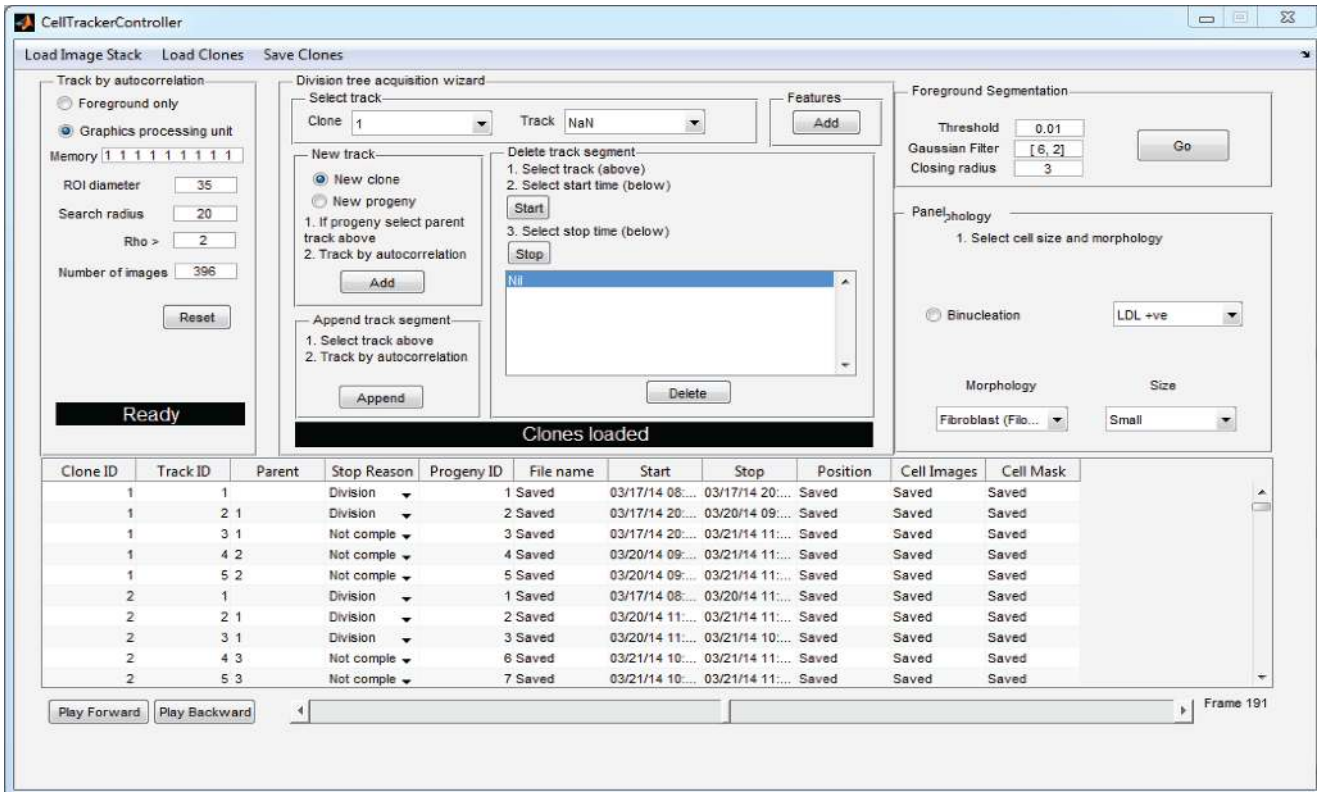



**Figure 24.9** Screenshot of NTT graphical user interface (GUI). The GUI allows control of cell tracking parameters such as region-of-interest (ROI) diameter, search radius, and correlation coefficient Rho. The GUI contains features for deleting and appending track segments as well as entering fate outcomes in a drop-down menu.

for each frame was referred to as a cell mask. To find the same cell in the subsequent frame, NTT searched all pixels within a user-defined search radius in the subsequent frame. A cross-correlation algorithm was then used to find the position of the cell mask within the search region that gave the highest correlated pixel intensity pattern. The search radius is the maximum radial distance in which to search for a cell mask in each frame. The default value of the search radius is 20 pixels, though this could be adjusted depending on cell velocity, density of the culture, and proximity to the edge of the FOV. Increasing and decreasing the search radius also respectively increased or decreased the time taken to find a cell mask in the subsequent frame. The correlation coefficient (Rho) was a commonly adjusted parameter in the cell tracking platform. Rho, a number between 0 and 1, defined the correlation between pixel patterns in consecutive frames that was required to find a match. If a correlation above the set threshold was found for two cell masks within consecutive frames the software automatically moved to the next frame and repeated. If correlation between pixel patterns in consecutive frames was below the threshold the software stopped and prompted the user to manually reposition the ROI over the cell nucleus. Rho was increased or decreased to optimize the accuracy and speed of tracking. If Rho was increased, the criteria for finding matching cells in consecutive frames was more stringent and, therefore, allowed for more accurate tracking. This is suitable for situations where cells are cultured at high density and do not have unique or distinctive features. If Rho was set >1, NTT operated in a manual tracking mode, where the user was required to reposition the ROI and double-click on each frame. If Rho was decreased, then the criteria for finding matching cells in consecutive frames was less stringent and cell tracking speed was increased. This was suitable when tracking cells at lower density and where cells had distinct features that did not change transiently.

This semiautomated method of tracking was almost error free, since all cell tracks were continuously observed as they were built and all cell event outcomes were visually identified and annotated. Division and death events were detected visually and recorded in a single cell pedigree structure along with the time of the observed event. If an error was encountered while tracking, the user stopped the software and deleted erroneous segments of a cell's track. The user entered all relevant and measureable data into a hierarchical database structure or single cell pedigree using a drop-down menu. $X$ and $Y$ positions of the center of the ROI (i.e., the center of a nucleus) were recorded for each frame, along with the time the image was acquired.

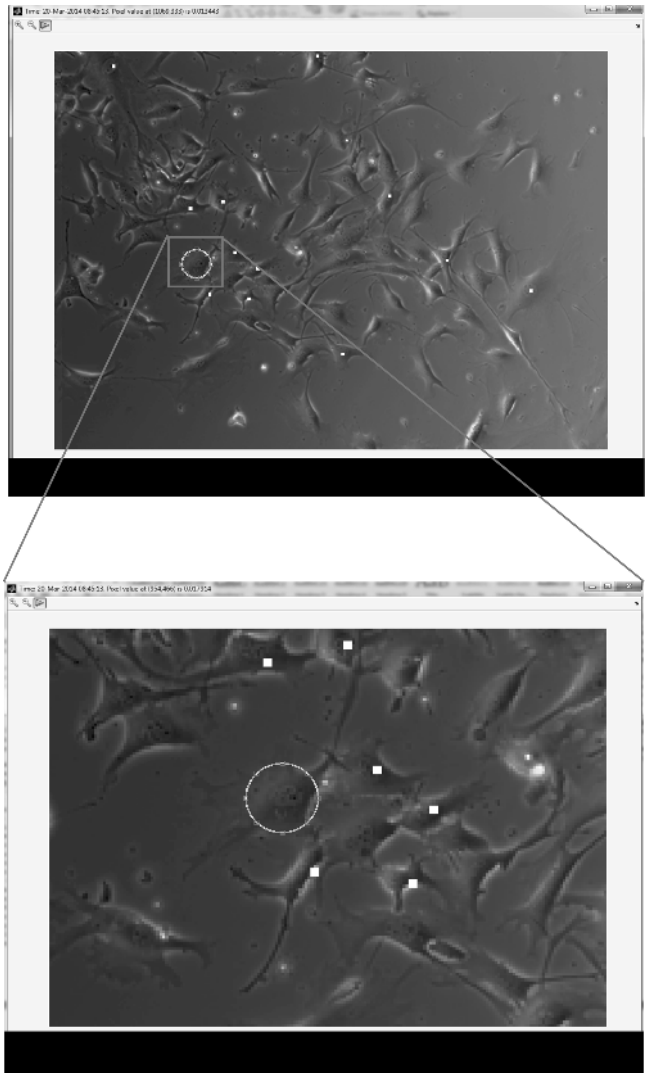


**Figure 24.10** Screenshots of NTT CellMovieViewer showing full FOV (top) and magnified region (bottom). The CellMovieViewer allows cells to be visualized while tracking. An adjustable ellipse is placed over the nucleus of the cell of interest as it is tracked through consecutive frames. White dots indicate tracked cells.

#### 24.4.2.6 Measuring fluoroscein isothiocyanate–acetylated low-density lipoprotein intensity

FITC–Ac-LDL intensity was measured for every cell at each time point using an extension of NTT developed for measuring reporter signals after cell tracking. Cell tracking produced a cell mask for every tracked cell in each frame. This mask contained pixel indices that formed a reference for the position of the cell's nucleus within each FOV. These pixel indices were used to determine the intensity of accumulated FITC–Ac-LDL by measuring the pixel intensities within the FITC channel. The cell mask provided a mapping reference to the cell nucleus. However, the FITC–Ac-LDL was accumulated in the perinuclear region and cytosol. Therefore, the cell mask was enlarged to record pixel intensities within a region surrounding the nucleus in the FITC channel. All measurements were manually validated after tracking by ensuring that the ROI was placed correctly over the cell nucleus at each time point. Once pixel intensities had been measured, the maximum and mean pixel intensities were calculated. The mean pixel intensity measurement was used for downstream analysis because intracellular staining was heterogeneous.

### 24.4.3 Results and discussion

#### 24.4.3.1 Defining cardiac colony-forming unit–fibroblast to endothelial cell fate transitions using acetylated low-density lipoprotein

Ac-LDL is known to be nonspecifically endocytosed by several cell types, including vascular smooth muscle cells, although at a slower rate than by ECs [90]. ECs can then only be identified by increased uptake of Ac-LDL compared with cell types in which endocytosis of the protein is nonspecific [90]. Therefore, to distinguish cells that had increased uptake (endothelial phenotype) from those that had nonspecific uptake (non-endothelial phenotype), a threshold of FITC–Ac-LDL intensity was established. The mean intensity of FITC–Ac-LDL accumulated within undifferentiated control cells, not exposed to differentiation conditions, was used for setting the threshold intensity. This threshold level of FITC–Ac-LDL was subtracted from FITC–Ac-LDL measurements for cCFUs exposed to EGM. Cells that had a mean FITC–Ac-LDL signal above the background level after thresholding were considered to have adopted an endothelial-like phenotype. Notably, the degree of FITC–Ac-LDL uptake in cells exposed to differentiation medium was higher than that observed for native murine aortic ECs.

However, there were differences in background fluorescence between control (DMEM) and EGM (IMDM) media because basal media were fluorescent, although at much lower levels compared with FITC–Ac-LDL. DMEM was observed to have a higher autofluorescence than IMDM, presumably due to a higher concentration of phenol red. Therefore, the mean FITC–Ac-LDL measurements were normalized to the intensity values at the beginning of the experiment for control and experimental groups.

Figure 24.11 shows the normalized mean Ac-LDL intensity measurements for control and differentiation conditions averaged over all cells. Clearly, the FITC–Ac-LDL signal in cells cultured in control conditions remained constant for the duration of the experiment. In contrast, in the first 4 days after exposure to EGM the mean FITC–Ac-LDL significantly increased to a peak level 6–10 times greater than that observed in control cells. This peak signal plateaued for some time before declining in intensity toward the end of the experiment. The reduction in mean intensity can be partly attributed to photobleaching of FITC–Ac-LDL. The time at which cells reached maximum uptake of Ac-LDL corresponded to the time at which cCFU-Fs changed from a spindle shaped to a cobblestone morphology (Figure 24.12, white arrows).

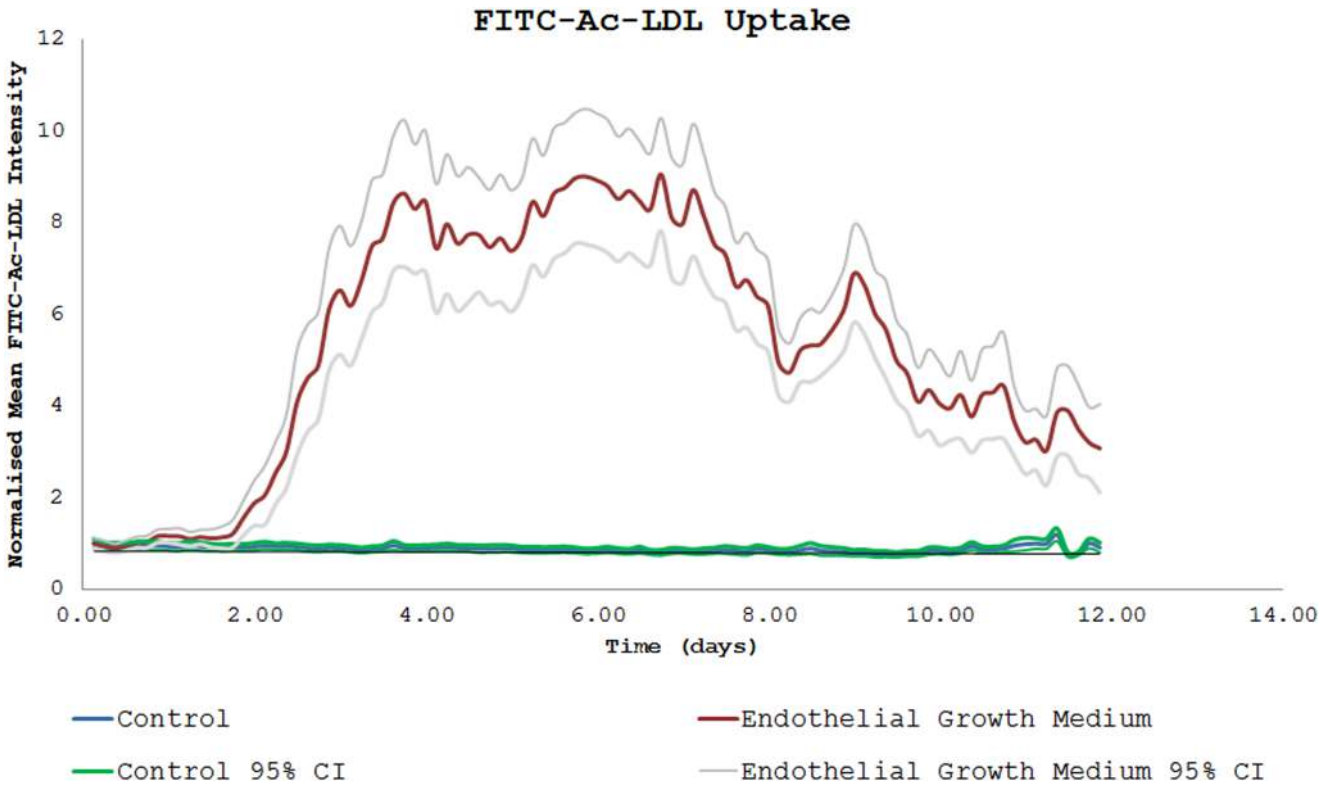



**Figure 24.11** Mean intensity of accumulated FITC-Ac-LDL (normalized to start of experiment) for cardiac CFU-F in control media and endothelial differentiation medium over 12 days in culture. Significant differences in mean intensity between control media and endothelial differentiation medium were observed. Bold black line and grey lines either side of bold black line, indicating peaking and plateauing of intensity of FITC-Ac-LDL accumulation at day 4 of culture, represent mean and 95% CI for cells grown in endothelial growth medium. The dashed line and the dotted lines either side of the dashed line running along the bottom of the figure represent mean and 95% CI for cells grown in control media.

#### 24.4.3.2 Quantifying cardiac colony-forming unit–fibroblast fate during endothelial differentiation

To analyze the effect of endothelial growth conditions on cCFU growth dynamics, the proportion of fate outcomes for Ac-LDL$^+$ and Ac-LDL$^-$ cells in both control and differentiation conditions were quantified (Figure 24.13). The fate outcomes recorded were division, death, lost, and incomplete. Incomplete fates occurred if the experiment ended before the final fate of the cell was observed. Lost cells and incomplete fates both resulted in right censoring; that is, no final fate was observed. In control conditions, all cells remained FITC–Ac-LDL$^-$ and therefore remained undifferentiated. In control cultures, approximately 38.7% divided, 9.9% died, 21.6% were lost, and 29.7% were incomplete. Figure 24.13b shows the quantification of fates for cells exposed to EGM. Approximately 53.5% of cells exposed to EGM became FITC–Ac-LDL$^+$, of which only 0.7% divided, 16.4% died, 5.7% were lost, and 30.7% were incomplete. In contrast, for the 46.5% of cells that remained FITC–Ac-LDL$^-$, 31.4% divided, 3.5% died, 10.7% were lost, and 0.7% were incomplete. Thus, distinct differences in the frequency of fate outcomes for control and differentiation cultures were observed. Furthermore, differential fate outcomes were observed for Ac-LDL$^+$ and Ac-LDL$^-$, suggesting that lineage commitment influenced the frequency of division and death. Interestingly, Ac-LDL$^+$ cells had a significantly lower frequency of division and a higher frequency of death compared with Ac-LDL$^-$ cells. It is important to note that in both control and differentiation conditions a significant number of cells were lost while tracking. Overlapping of cell trajectories was the main cause of lost cells, as it was impossible to distinguish one cell from another. This could be attributed to the high density of the culture, which was selected to achieve optimal conditions for endothelial differentiation.

As previously mentioned, analyses of cell fate outcomes revealed significant differences in the frequency of division and death for Ac-LDL$^-$ and Ac-LDL$^+$ cells. To further investigate these differences, survival analysis was employed to estimate rates of division and death in each group. Competing risks statistics, a type of survival analysis, were used to estimate cumulative incidence functions (CIFs) for division and death in each group [93]. The cumulative incidence of division can be defined as the probability that a cell has divided before a given time. To test for statistically significant differences between CIFs for each group, a *K*-sample test was applied. Figure 24.14 shows division and death CIFs for Ac-LDL$^-$ and Ac-LDL$^+$ cells. cCFUs that had become Ac-LDL$^+$ exhibited significantly reduced probability of division and a significantly increased probability of death compared with Ac-LDL$^-$ cells.

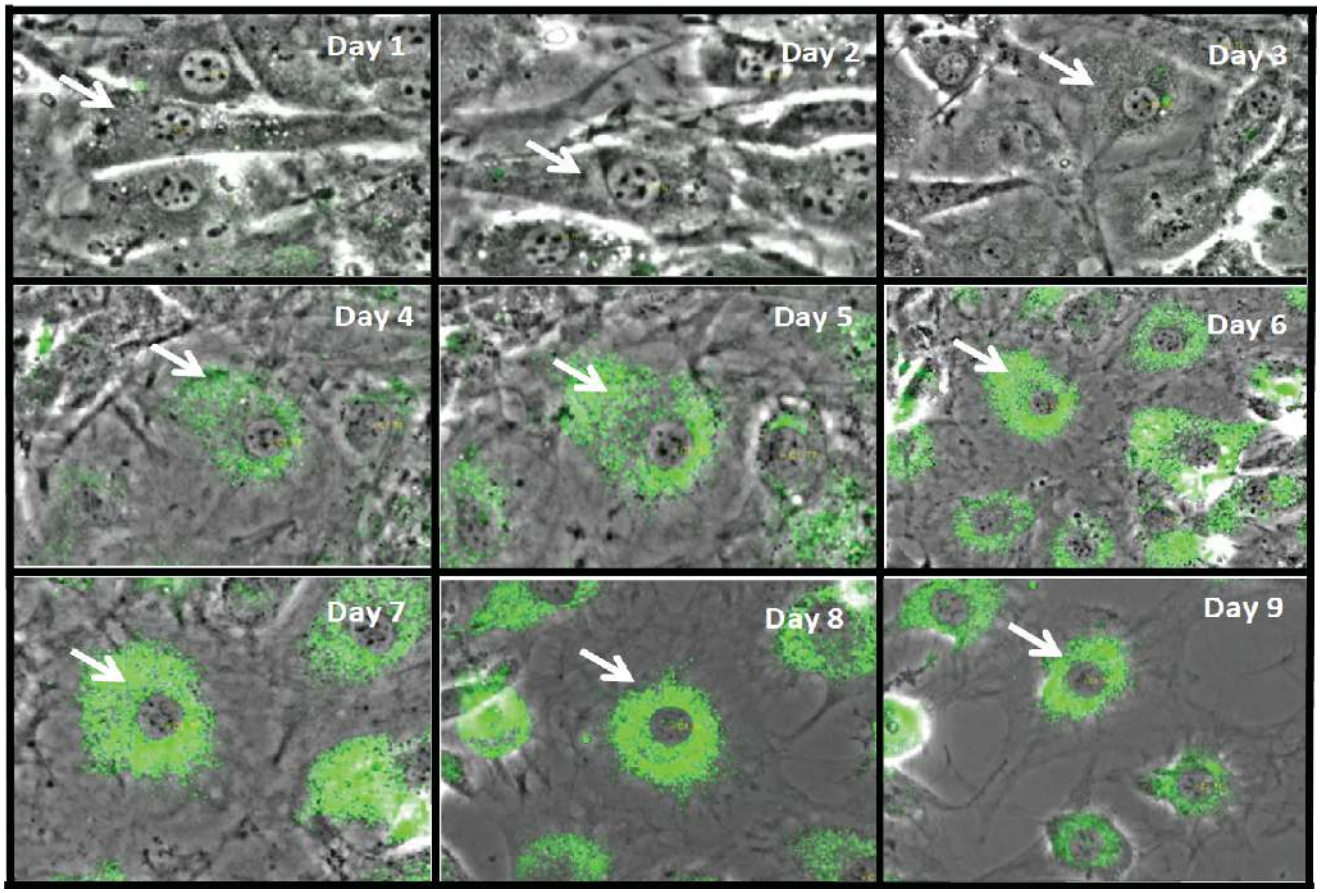



**Figure 24.12** Phase-contrast images (20×) showing transition of a cardiac CFU-F from an LDL$^-$ cell with a mesenchymal morphology to an LDL$^+$ cell with cobblestone morphology after treatment with EGM for 9 days (white arrows). Day 1 shows a typical cCFU-F with canonical MSC morphology (spindle shaped). After 4 days of exposure to EGM the cCFU-F changes to a round, flattened cell that begins to show signs of contact inhibition and intracellular accumulation of LDL. (See color plate section.)

### 24.4.4 Conclusion and future work

The dynamics of cCFU-F to EC fate transitions was studied at the single cell level by live cell imaging and single cell tracking. These experiments confirmed previous reports suggesting that cCFU-Fs differentiate into the EC lineage using fluorescently labeled Ac-LDL as a differentiation marker [23]. This example shows how endothelial fate is coupled to marked changes in cell cycle dynamics and death. A significant proportion of Ac-LDL$^-$ cells differentiated into Ac-LDL$^+$ cells without an increased rate of apoptosis of Ac-LDL$^-$ cells. Thus, VEGF directs differentiation rather than acting as a permissive survival factor that prevents apoptosis of cells not destined to become ECs (i.e., restricted endothelial progenitor cells). EGM also directs cell cycle arrest with an increased rate of cell death once cells become Ac-LDL$^+$.

Uptake of Ac-LDL alone is clearly not sufficient to prove that these are ECs. This would need to be confirmed by *in vitro* vascular network formation. While Ac-LDL uptake is an established assay for identification of ECs, additional markers such as PECAM (CD31) and VE-cadherin would need to be included to extend this study, though there are inconsistent reports on the expression of these mature EC markers by MSC-derived ECs [83,87–89]. Furthermore, we have not studied the autocrine effect of MSCs in this culture system. Future studies should examine the effect of seeding density on EC differentiation since MSCs themselves are probably secreting cytokines that modulate EC production. Future work should also include improvements to technical aspects of the study, such as continuous replacement of media and FITC–Ac-LDL to eliminate batch-to-batch variations in cell growth and Ac-LDL uptake measurements.

This study shows how MSC cultures can be directed to form ECs, which could provide a new strategy for revascularization therapy. A fascinating extension of this study would be to combine live cell imaging and single cell tracking with single cell gene expression analyses to study gene expression signatures associated with cCFU to EC transitions.

## 24.5 Future perspective on live cell imaging and single cell tracking

Current software packages for image acquisition, image management, cell tracking, and analysis and visualization tools fall

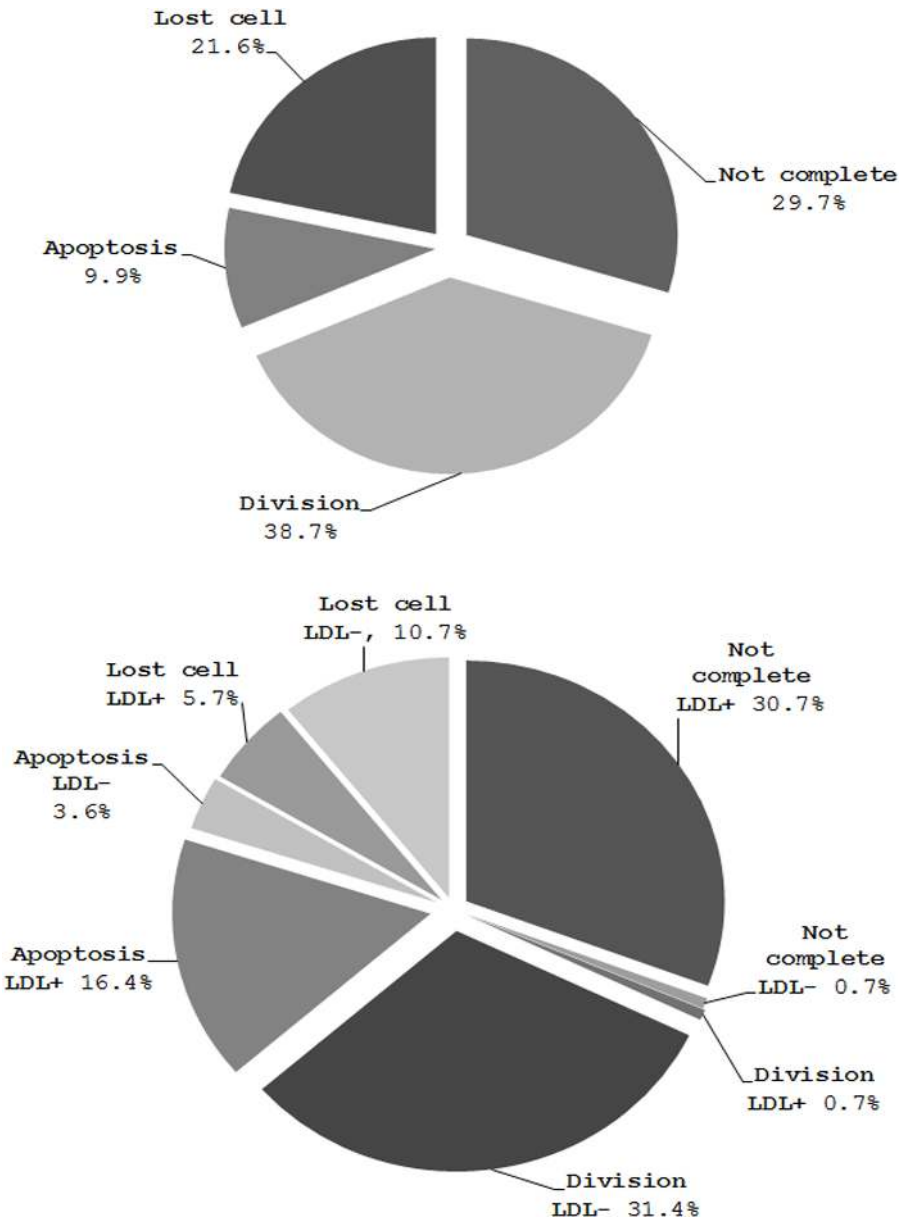



**Figure 24.13** Pie charts showing proportion of fate outcomes for cCFU-Fs cultured in control medium (a) and cCFU-Fs cultured in EGM (b). Significantly different proportions of fate outcomes were observed for control and differentiation conditions, as well as for $LDL^-$ and $LDL^+$ cells.

short of providing a user-friendly environment for most biologists to utilize live cell imaging and single cell tracking. Open-source software packages will enable software designers, statisticians, and biologists to interact and continually improve the analytical power of software. Increasing automation will remove the cell trajectory mapping bottleneck, reducing the time required to accurately track cells and annotate pedigrees. These platforms should aim to be user friendly, developer friendly, modular, validated to ensure accuracy and consistency of results, and compatible with different operating systems [94]. Appropriate and tractable statistical methodology is absolutely necessary for unbiased interpretation of cell lifetime analysis. Therefore, to maintain scientific rigor, appropriate statistical methodology should be enforced by peer review [95].

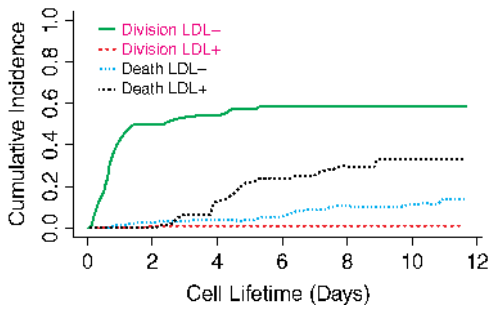



**Figure 24.14** Division and death cumulative incidence functions (CIFs) for $LDL^-$ and $LDL^+$ cells. Cumulative incidence of division was significantly higher for $LDL^-$ cells (top line) relative to $LDL^+$ cells (bottom line) ($p$ = 1:78E−15). Cumulative incidence of death was significantly lower for $LDL^-$ cells (second line from bottom) compared with $LDL^+$ cells (third line from bottom) ($p$ = 9:85E−04).

Application of live cell imaging and single cell analysis to the study of MSC biology will help address many of the methodological problems facing this research field. In contrast to phenotypically well-defined hierarchical differentiation systems such as hematopoiesis, MSC differentiation hierarchy and plasticity is poorly defined at the molecular level. Even though sets of markers to define the human MSC system have been adopted for verification of clinical MSC products [96], the lack of surface markers to identify different cell states within the MSC hierarchy is impeding progress. These should identify long-term self-renewing MSCs, transit amplifying or multipotent populations, and lineage-committed cell types.

Navigating the MSC lineage map by directing extrinsic and intrinsic signals will be made possible by discovery and development of surface markers or reporter fluorescent proteins that identify MSC fate transitions. These developments, when combined with the growing fields of single cell gene expression analysis and microfluidic technologies for single cell analysis, will usher in a new age of biological inquiry, where one is able to dissect the functional role of single molecules in single cells in real time.

## References


1 A. Raj, A. van Oudenaarden. Nature, nurture, or chance: stochastic gene expression and its consequences. *Cell* 2008;**135** (2):216–226.
2 O.J. Rando, K.J. Verstrepen. Timescales of genetic and epigenetic inheritance. *Cell* 2007;**128** (4):655–668.
3 A. Kuchina, L. Espinar, T. Çağatay, *et al.* Temporal competition between differentiation programs determines cell fate choice. *Mol Syst Biol* 2011;**7** (1):557.
4 D.L. Coutu, T. Schroeder. Probing cellular processes by long-term live imaging – historic problems and current solutions. *J Cell Sci* 2013;**126** (17):3805–3815.
5 K.D. Kokkaliaris, D. Loeffler, T. Schroeder. Advances in tracking hematopoiesis at the single-cell level. *Curr Opin Hematol* 2012;**19** (4):243–249.
6 T. Schroeder. Long-term single-cell imaging of mammalian stem cells. *Nat Methods* 2011;**8** (4s):S30–S35.
7 M. Pevsner-Fischer, S. Levin, D. Zipori. The origins of mesenchymal stromal cell heterogeneity. *Stem Cell Rev Rep* 2011;**7** (3):560–568.
8 D.G. Phinney. Functional heterogeneity of mesenchymal stem cells: implications for cell therapy. *J Cell Biochem* 2012;**113** (9):2806–2812.
9 K. Sivasubramaniyan, D. Lehnen, R. Ghazanfari, *et al.* Phenotypic and functional heterogeneity of human bone marrow- and amnion-derived MSC subsets. *Ann N Y Acad Sci* 2012;**1266** (1):94–106.
10 T. Schroeder. Imaging stem-cell-driven regeneration in mammals. *Nature* 2008;**453** (7193):345–351.
11 G.A. Dunn, G.E. Jones. Cell motility under the microscope: Vorsprung durch Technik. *Nat Rev Mol Cell Biol* 2004;**5** (8):667–672.
12 M.J. Whitfield, W.C.J. Lee, K.J. Van Vliet. Onset of heterogeneity in culture-expanded bone marrow stromal cells. *Stem Cell Res* 2013;**11** (3):1365–1377.
13 L. Xiang, R.W. Chan, E.H. Ng, W.S. Yeung. Nanoparticle labeling identifies slow cycling human endometrial stromal cells. *Stem Cell Res Ther* 2014;**5** (4):84.
14 J.T. Koepsel, S.G. Loveland, M.P. Schwartz, *et al.* A chemically-defined screening platform reveals behavioral similarities between primary human mesenchymal stem cells and endothelial cells. *Integr Biol (Camb)* 2012;**4** (12):1508–1521.
15 Z. Zhao, C. Watt, A. Karystinou, *et al.* Directed migration of human bone marrow mesenchymal stem cells in a physiological direct current electric field. *Eur Cells Mater* 2011;**22**:344–358.
16 A.M. Altman, V. Gupta, C.N. Ríos, *et al.* Adhesion, migration and mechanics of human adipose-tissue-derived stem cells on silk fibroin–chitosan matrix. *Acta Biomater* 2010;**6** (4):1388–1397.
17 H.M. Eilken, S.-I. Nishikawa, T. Schroeder. Continuous single-cell imaging of blood generation from haemogenic endothelium. *Nature* 2009;**457** (7231):896–900.
18 M.R. Costa, F. Ortega, M.S. Brill, *et al.* Continuous live imaging of adult neural stem cell division and lineage progression *in vitro*. *Development* 2011;**138** (6):1057–1068.
19 B. Dykstra, J. Ramunas, D. Kent, *et al.* High-resolution video monitoring of hematopoietic stem cells cultured in single-cell arrays identifies new features of self-renewal. *Proc Natl Acad Sci U S A* 2006;**103** (21):8185–8190.
20 M.A. Rieger, P.S. Hoppe, B.M. Smejkal, *et al.* Hematopoietic cytokines can instruct lineage choice. *Science* 2009;**325**:217–218.
21 K.R. Duffy, P.D. Hodgkin. Intracellular competition for fates in the immune system. *Trends Cell Biol* 2012;**22** (9):457–464.
22 M.R. Dowling, A. Kana, S. Heinzela, *et al.* Stretched cell cycle model for proliferating lymphocytes. *Proc Natl Acad Sci U S A* 2014;**111** (17):6377–6382.
23 J.J.H. Chong, V. Chandrakanthan, M. Xaymardan, *et al.* Adult cardiac-resident MSC-like stem cells with a proepicardial origin. *Cell Stem Cell* 2011;**9** (6):527–540.
24 C. Terryn, A. Bonnomet, J. Cutrona, *et al.* Video-microscopic imaging of cell spatio-temporal dispersion and migration. *Crit Rev Oncol Hematol* 2009;**69** (2):144–152.
25 D. Murphy. *Fundamentals of Light Microscopy and Electronic Imaging*. John Wiley & Sons Canada Ltd, 2001.
26 H. Eilken, M. Rieger, P. Hoppe, *et al.* Continuous long-term detection of live cell surface markers by "in culture" antibody staining. *Protoc Exch* 2011. doi: 10.1038/protex.2011.205.
27 M. Collins, P. Gunst, B. Muller-Borer. Functional integration of quantum dot labeled mesenchymal stem cells in a cardiac microenvironment. In: A. Fontes, B.S. Santos (eds), *Quantum Dots: Applications in Biology*. Methods in Molecular Biology, vol. **1199**. Springer: New York, 2014; pp. 141–154.
28 X. Liao, S. Lu, Y. Wu, *et al.* The effect of differentiation induction on FAK and Src activity in live HMSCs visualized by FRET. *PLoS ONE* 2013;**8** (8):e72233.
29 I.H. Huang, C.T. Hsiao, J.C. Wu, *et al.* GEF-H1 controls focal adhesion signaling that regulates mesenchymal stem cell lineage commitment. *J Cell Sci* 2014;**127** (19):4186–4200.
30 T. Zimmermann, J. Rietdorf, R. Pepperkok, Spectral imaging and its applications in live cell microscopy. *FEBS Lett* **546** (1):87–92.
31 D.J. Stephens, V.J. Allan. Light microscopy techniques for live cell imaging. *Science* 2003;**300** (5616):82–86.
32 R. Yuste. Fluorescence microscopy today. *Nat Methods* 2005;**2** (12):902–94.
33 G.S.L. Teo, J.A. Ankrum, R. Martinelli, S+++*et al.* Mesenchymal stem cells transmigrate between and directly through TNF-α-activated endothelial cells. *Stem Cells* 2012;**30** (11):2472–2486.

34 T.P. Lozito, R.S. Tuan. Endothelial and cancer cells interact with mesenchymal stem cells via both microparticles and secreted factors. *J Cell Molr Med* 2014;**18** (12):2372–2384.

35 L. Kucerova, S. Skolekova, L. Demkova, *et al.* Long-term efficiency of mesenchymal stromal cell-mediated CD-MSC/5FC therapy in human melanoma xenograft model. *Gene Ther* 2014;**21** (10):874–887.

36 K. Li, E.D. Miller, M. Chen, *et al.* Cell population tracking and lineage construction with spatiotemporal context. *Med Image Anal* 2008;**12** (5):546–566.

37 J.N. Harada, K.E. Bower, A.P. Orth, *et al.* Identification of novel mammalian growth regulatory factors by genome-scale quantitative image analysis. *Genome Res* 2005;**15** (8):1136–1144.

38 K. Kulangara, Y. Yang, J. Yang, K.W. Leong. Nanotopography as modulator of human mesenchymal stem cell function. *Biomaterials* 2012;**33** (20):4998–5003.

39 A.M. Silva, M.I. Oliveira, L. Sette, *et al.* Resveratrol as a natural anti-tumor necrosis factor-α molecule: implications to dendritic cells and their crosstalk with mesenchymal stromal cells. *PLoS ONE* 2014;**9** (3):e91406.

40 L. Kamentsky, T.R. Jones, A. Fraser, *et al.* Improved structure, function and compatibility for CellProfiler: modular high-throughput image analysis software. *Bioinformatics* 2011;**27** (8): 1179–1180.

41 A.E. Carpenter, T.R. Jones, M.R. Lamprecht, *et al.* CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol* 2006;**7** (10):R100.

42 F.D. Sigoillot, J.F. Huckins, F. Li, *et al.* A time-series method for automated measurement of changes in mitotic and interphase duration from time-lapse movies. *PLoS ONE* 2011;**6** (9):e25511.

43 A.-A. Liu, K. Li, T. Kanade. A semi-Markov model for mitosis segmentation in time-lapse phase contrast microscopy image sequences of stem cell populations. *IEEE Trans Med Imaging* 2012;**31** (2):359–369.

44 S.G. Ball, A. Shuttleworth, C.M. Kielty. Inhibition of platelet-derived growth factor receptor signaling regulates Oct4 and Nanog expression, cell shape, and mesenchymal stem cell potency. *Stem Cells* 2012;**30** (3):548–560.

45 N. Scherf, K. Franke, I. Glauche, *et al.* On the symmetry of siblings: automated single-cell tracking to quantify the behavior of hematopoietic stem cells in a biomimetic setup. *Exp Hematol* 2012;**40** (2):119–130.

46 P. Bogdan, B.M. Deasy, B. Gharaibeh, *et al.* Heterogeneous structure of stem cells dynamics: statistical models and quantitative predictions. *Sci Rep* 2014;**4**:4826.

47 H.V. Desai, I.S. Voruganti, C. Jayasuriya, *et al.* Live-cell, temporal gene expression analysis of osteogenic differentiation in adipose-derived stem cells. *Tissue Eng Part A* 2013;**19**(1–2):40–48.

48 B. Muller-Borer, M. Collins, P. Gunst, *et al.* Quantum dot labeling of mesenchymal stem cells. *J Nanobiotechnol* 2007;**5** (1):9.

49 N.C. Shaner, P.A. Steinbach, R.Y. Tsien. A guide to choosing fluorescent proteins. *Nat Methods* 2005;**2** (12):905–909.

50 C.R. Parish. Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunol Cell Biol* 1999;**77** (6):499–508.

51 R.E. Nordon, S.S. Ginsberg, C.J. Eaves. High-resolution cell division tracking demonstrates the Flt3-ligand-dependence of human marrow $CD34^{+}CD38^{-}$ cell production *in vitro*. *Br J Haematol* 1997;**98** (3):528–539.

52 K. Ko, R. Odell, R.E. Nordon. Analysis of cell differentiation by division tracking cytometry. *Cytometry A* 2007;**71**:773–782.

53 H. Polzer, E. Volkmer, M.M. Saller, *et al.* Long-term detection of fluorescently labeled human mesenchymal stem cell *in vitro* and *in vivo* by semi-automated microscopy. *Tissue Eng Part C Methods* 2011;**18** (2):156–165.

54 A.B. Lyons. Divided we stand: tracking cell proliferation with carboxyfluorescein diacetate succinimidyl ester. *Immunol Cell Biol* 1999;**77** (6):509–515.

55 R.E. Nordon, N. Nakamura, C. Ramirez, R. Odell. Analysis of growth kinetics by division tracking. *Immunol Cell Biol* 1999;**77**:523–529.

56 G. Chamberlain, H. Smith, G.E. Rainger, J. Middleton. Mesenchymal stem cells exhibit firm adhesion, crawling, spreading and transmigration across aortic endothelial cells: effects of chemokines and shear. *PLoS ONE* 2011;**6** (9):e25663.

57 E. Eggenhofer, V. Benseler, A. Kroemer, *et al.* Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Front Immunol* 2012;**3**:297.

58 R.R. Taghizadeh, J.L. Sherley. CFP and YFP, but not GFP, provide stable fluorescent marking of rat hepatic adult stem cells. *J Biomed Biotechnol* 2008;**2008**:453590.

59 N. Kaukua, M.K. Shahidi, C. Konstantinidou, *et al.* Glial origin of mesenchymal stem cells in a tooth model system. *Nature* 2014;**513** (7519):551–554.

60 M.J. Go, C. Takenaka, H. Ohgushi. Effect of forced expression of basic fibroblast growth factor in human bone marrow-derived mesenchymal stromal cells. *J Biochem* 2007;**142** (6):741–748.

61 G.-J. Kremers, S.G. Gilbert, P.J. Cranfill, *et al.* Fluorescent proteins at a glance. *J Cell Sci* 2011;**124** (2):157–160.

62 M. Walling, J. Novak, J.R.E. Shepard. Quantum dots for live cell and *in vivo* imaging. *Int J Mol Sci* 2009;**10** (2):441–491.

63 B. Shah, J. Mao. Labeling of mesenchymal stem cells with bioconjugated quantum dots. In: K. Shah (ed.), *Molecular Imaging. Methods in Molecular Biology*, vol. **680**.Humana Press, 2011; pp. 61–75.

64 M. Rutten, M. Janes, B. Laraway, *et al.* Comparison of quantum dots and CM-DiI for labeling porcine autologous bone marrow mononuclear progenitor cells. *Open Stem Cell J* 2010;**2**:25–36.

65 O. Seleverstov, J.M. Phang, O. Zabirnyk. Semiconductor nanocrystals in autophagy research: methodology improvement at nanosized scale. In: J.K. Daniel (ed.), *Methods in Enzymology*, vol. **452**. Academic Press, 2009; pp. 277–296.

66 M.J. Ruedas-Rama, J.D. Walters, A. Orte, E.A.H. Hall. Fluorescent nanoparticles for intracellular sensing: a review. *Anal Chim Acta* 2012;**751**:1–23.

67 A.E. Prigodich, P.S. Randeria, W.E. Briley, *et al.* Multiplexed nanoflares: mRNA detection in live cells. *Anal Chem* 2012;**84** (4):2062–2066.

68 B. Goldman. Embryonic stem cells 2.0. *Nat Rep Stem Cells* 2008. doi: 10.1038/stemcells.2008.67.

69 A.P. Croft, S.A. Przyborski. Formation of neurons by non-neural adult stem cells: potential mechanism implicates an artifact of growth in culture. *Stem Cells* 2006;**24** (8):1841–1851.

70 P. Lu, A. Blesch, M.H. Tuszynski. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *J Neurosci Res* 2004;**77** (2):174–191.

71 D.E. Discher, D.J. Mooney, P.W. Zandstra. Growth factors, matrices, and forces combine and control stem cells. *Science* 2009;**324** (5935):1673–1677.

72 V.H. Fan, A. Au, K. Tamama, *et al.* Tethered epidermal growth factor provides a survival advantage to mesenchymal stem cells. *Stem Cells* 2007;**25** (5):1241–1251.

73 J. Fu, Y.-K. Wang, M.T. Yang, *et al.* Mechanical regulation of cell function with geometrically modulated elastomeric substrates. *Nat Methods* 2010;**7** (9):733–736.

74 S.-H. Hsu, G.-S. Huang. Substrate-dependent Wnt signaling in MSC differentiation within biomaterial-derived 3D spheroids. *Biomaterials* 2013;**34** (20):4725–4738.

75 Z. Liu, L. Xiao, B. Xu, *et al.* Covalently immobilized biomolecule gradient on hydrogel surface using a gradient generating microfluidic device for a quantitative mesenchymal stem cell study. *Biomicrofluidics* 2012;**6** (2):024111–024111-12.

76 H.-W. Wu, C.-C. Lin, G.-B. Lee. Stem cells in microfluidics. *Biomicrofluidics* 2011;**5** (1):013401.

77 H. Chen, J.A. Cornwell, H. Zhang, *et al.* Cardiac-like flow generator for long-term imaging of endothelial cell responses to circulatory pulsatile flow at microscale. *Lab Chip* 2013;**13** (15):2999–3007.

78 S. Gobaa, S. Hoehnel, M. Roccio, *et al.* Artificial niche microarrays for probing single stem cell fate in high throughput. *Nat Methods* 2011;**8** (11):949–955.

79 A.J. Engler, S. Sen, H.L. Sweeney, D.E. Discher. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126** (4):677–689.

80 J.R. Tse, A.J. Engler. Stiffness gradients mimicking *in vivo* tissue variation regulate mesenchymal stem cell fate. *PLoS ONE* 2011;**6** (1): e15978.

81 R. Peerani, B.M. Rao, C. Bauwens, *et al.* Niche-mediated control of human embryonic stem cell self-renewal and differentiation. *EMBO J* 2007;**26** (22):4744–4755.

82 D.M. Reed, G. Foldes, S.E. Harding, J.A. Mitchell. Stem cell-derived endothelial cells for cardiovascular disease: a therapeutic perspective. *Br J Clin Pharmacol* 2013;**75** (4):897–906.

83 J.W. Liu, S. Dunoyer-Geindre, V. Serre-Beinier, *et al.* Characterization of endothelial-like cells derived from human mesenchymal stem cells. *J Thromb Haemost* 2007;**5** (4):826–834.

84 K. Janeczek Portalska, A. Leferink, N. Groen, *et al.* Endothelial differentiation of mesenchymal stromal cells. *PLoS ONE* 2012;**7** (10):e46842.

85 Y. Cao, Z. Sun, L. Liao, *et al.* Human adipose tissue-derived stem cells differentiate into endothelial cells *in vitro* and improve postnatal neovascularization *in vivo*. *Biochem Biophys Res Commun* 2005;**332** (2):370–379.

86 L.J. Fischer, S. McIlhenny, T. Tulenko, *et al.* Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. *J Surg Res* 2009;**152** (1):157–166.

87 J. Oswald, S. Boxberger, B. Jørgensen, *et al.* Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 2004;**22** (3):377–384.

88 D. Liu, X. Zhang, X. Li, *et al.* Differentiation of the human marrow mesenchymal stem cells into vascular endothelium-like cells *in vitro*. In: Y. Peng, X. Weng (eds), *7th Asian-Pacific Conference on Medical and Biological Engineering*. IFMBE Proceedings, vol. **19**.Springer: Berlin, 2008; pp. 80–83.

89 P. Au, J. Tam, D. Fukumura, R.K. Jain. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 2008;**111** (9):4551–4558.

90 J.C. Voyta, D.P. Via, C.E. Butterfield, B.R. Zetter. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 1984;**99** (6): 2034–2040.

91 K.K. Sørensen, J. Melkko, B. Smedsrød. Scavenger-receptor-mediated endocytosis in endocardial endothelial cells of Atlantic cod *Gadus morhua*. *J Exp Biol* 1998;**201** (11):1707–1718.

92 J.-D. Dong, Y.-Q. Gu, C.-M. Li, *et al.* Response of mesenchymal stem cells to shear stress in tissue-engineered vascular grafts. *Acta Pharmacol Sin* 2009;**30** (5):530–536.

93 T. Scheike, M.-J. Zhang. Analyzing competing risks data using the R timereg package. *J Stat Softw* 2011;**38** (2):1–15.

94 A.E. Carpenter, L. Kamentsky, K.W. Eliceiri. A call for bioimaging software usability. *Nat Methods* 2012;**9** (7):666–670.

95 A. Cardona, P. Tomancak., Current challenges in open-source bioimage informatics. *Nat Methods* 2012;**9** (7):661–665.

96 M. Dominici, K.L. Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8** (4):315–317.

CHAPTER 25

# The role of mesenchymal stem/stromal cells in angiogenesis

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## 25.1 Introduction

Blood vessels are indispensable as they deliver oxygen and essential nutrients to nearly every organ of the body. Therefore, it is not surprising that inadequate blood vessel growth and function contribute to a wide variety of diseases, such as myocardial infarction, stroke, chronic wounds, and neurodegeneration [1]. Over the past decades our understanding of the molecular mechanisms of angiogenesis (blood vessel formation) has increased exponentially and has resulted in a number of new therapeutic strategies. So far, however, these new approaches to improve blood vessel growth have had only limited success as they have mainly focused on gene therapy or the administration of recombinant proangiogenic proteins and small molecules [2]. New cell-based treatments, and especially mesenchymal stem/stromal cell (MSC) therapies, are currently being investigated as an option to enhance neovascularization. There is increasing interest in understanding and enhancing the angiogenic capability of MSCs. This chapter summarizes the current knowledge on the process of angiogenesis and provides an overview of the angiogenic actions of MSCs *in vitro* and *in vivo*. Additionally, we will discuss ongoing preclinical and clinical trials with MSCs in pathologies caused by limited blood vessel formation, such as stroke and myocardial infarction.

## 25.2 The current concept of angiogenesis

Blood vessels form extensive networks that supply oxygen and nutrients to every part of the body, dispose of waste metabolites, and allow the patrolling of immune cells. The importance of the blood vessel network is illustrated by its emergence in the early phases of the embryonic development. In vertebrates the circulatory system is the first functional organ to arise and is indispensable for nourishing the rapidly developing tissues. The vasculature is mainly formed through three cellular processes: vasculogenesis, angiogenesis, and arteriogenesis (Figure 25.1).

First, vasculogenesis occurs. This is the *de novo* formation of a basic vascular system from the mesoderm-derived endothelial

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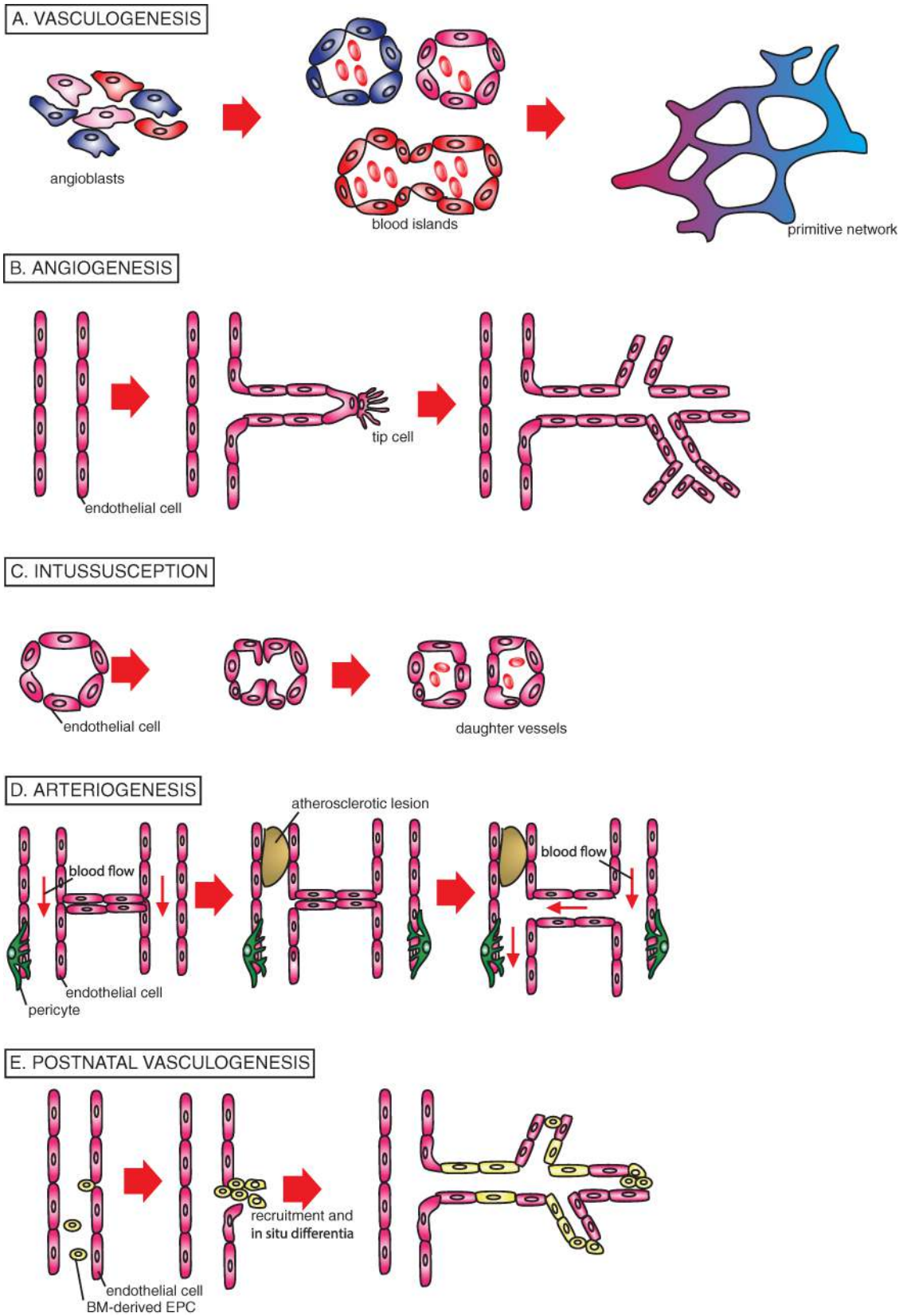



**Figure 25.1** Different types of blood vessel growth. (A) Vasculogenesis is defined as the growth of a primitive vascular network out of precursory cells, the angioblasts. (B) Angiogenesis is the growth of new blood capillaries sprouting from preexisting blood vessels. In intussusceptive angiogenesis (C) the capillary wall extends toward the lumen, splitting the blood vessel in two, resulting in two new daughter capillaries. (D) Arteriogenesis occurs mostly in adult organisms as a reaction to atherosclerotic plaques: preexisting collateral arterioles expand and form large blood vessels that are able to compensate for the loss of function of occluded arteries. (E) In adult vasculogenesis, endothelial precursory cells (EPCs) are recruited from the bone marrow (BM) followed by *in situ* differentiation, migration, and incorporation into the blood vessels.

precursor, the angioblast (Figure 25.1A) [3,4]. Second, this vascular plexus is dramatically expanded during angiogenesis, which is defined as the growth of new blood capillaries sprouting from preexisting blood vessels (Figure 25.1B) [5–7]. Finally, blood vessel maturation takes place when the endothelial cells (ECs) are covered by pericytes or smooth muscle cells, or both, to provide stability and regulate perfusion [4,8]. In the adult organism, blood vessels mostly develop through angiogenesis; but there are other mechanisms, such as the splitting of preexisting vessels, known as intussusception (Figure 25.1C), growth or increase of functional collateral arteries from preexisting anastomoses known as arteriogenesis (Figure 25.1D), or by the incorporation of circulating precursor cells into vessel structures, for example postnatal vasculogenesis (Figure 25.1E) [9–11].

Histologically, the inner wall of a blood vessel consists of a monolayer of ECs, which are surrounded by a basement membrane, extracellular matrix (ECM) and mural cells such as pericytes. This coat of mural cells and basement membrane prevent resident ECs from leaving their positions. In adults, blood vessels and ECs remain in a quiescent state and are rarely activated to form new branches. It is only in response to specific stimulation, such as hypoxia or inflammation, that the angiogenic process is initiated [1].

The first step of angiogenesis is local destruction of the basement membrane and dissociation of the mural cells to liberate the ECs (Figure 25.2A). Matrix metalloproteinases play an important role in this step. They degrade the basement membrane and ECM, thereby not only liberating the ECs but also releasing proangiogenic factors that are stored in the ECM. Next, the ECs proliferate, migrate toward the angiogenic stimuli, and form a primitive tube (Figure 25.2). For more than a decade now it has been clear that not all activated ECs play the same role in these phases of angiogenesis. A specialized endothelial tip cell, which protrudes filopodia upon activation, guides and coordinates the direction of the branch formation. It is followed by highly proliferative endothelial stalk cells [12,13]. These stalk cells form the lumen of the primitive blood vessel, either by the fusion of intracellular pinocytic vesicles or by rearrangement of their shape and junctions [4,14]. Next, the tip cell anastomoses with tip cells from adjacent sprouts and a primitive vessel loop is built that allows blood to flow through it. Finally, blood vessel maturation is established: ECs resume their quiescent state and again become covered with pericytes and new basement membrane. EC junctions are established to ensure optimal blood perfusion [15]. This maturation step is vital for the survival of the blood vessel as immature blood

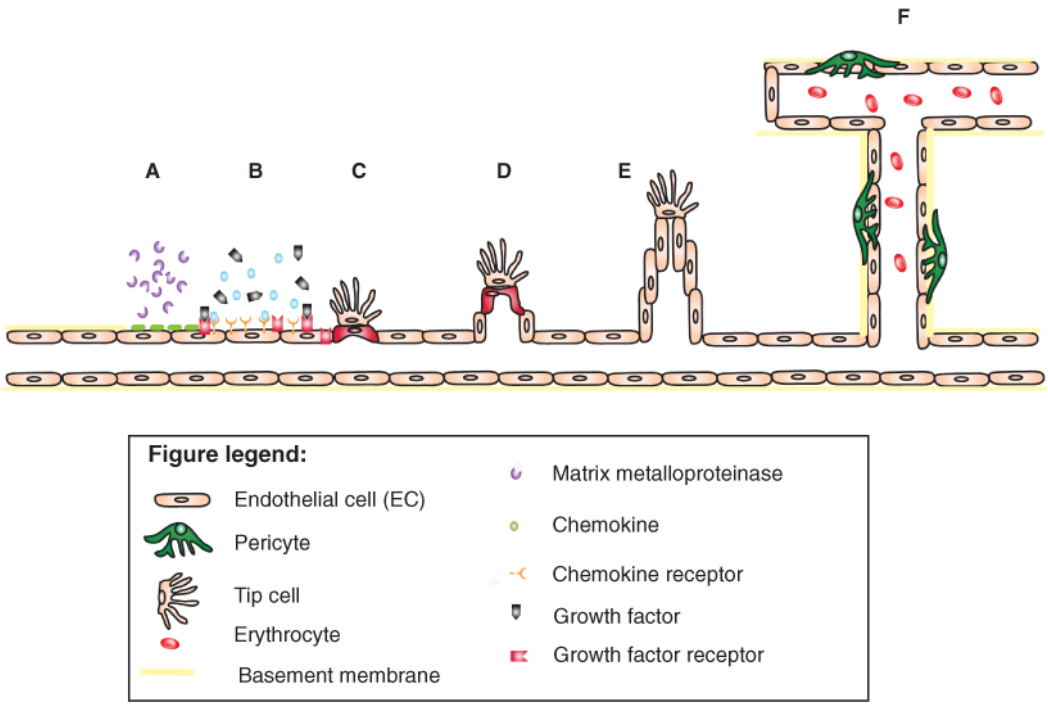



**Figure 25.2** Key steps of angiogenesis. Small blood vessels consist of hollow tubes aligned with ECs surrounded by pericytes, a basement membrane, and ECM. Angiogenesis is a multistep process involving the following steps: (A) breakdown of the ECM and basement membrane by, for example, matrix metalloproteinases. (B) EC activation by angiogenic factors, such as growth factors and cytokines. (C–E) Formation of a tip cell that guides the migration and a stack cell that proliferates and forms a primitive tube that matures into a fully functional blood vessel by pericyte attachment and deposition of ECM and basement membrane (F).

vessels are unstable and rapidly regress [4]. Observations of Carmeliet and colleagues revealed that the metabolism of the ECs is altered during angiogenesis. After stimulation with vascular endothelial growth factor (VEGF), glycolysis is induced in the ECs and is higher in tip cells than in stalk cells. The glycolytic activator phosphofructokinase-2/fructose-2,6-bisphosphatase 3 has been identified as playing a key role in EC proliferation and motility *in vitro* as well as in blood vessel growth *in vivo* in zebrafish embryos and the postnatal mouse retina [16–18].

For over 40 years now scientists have been trying to fully understand the process of normal and pathological angiogenesis. It is now clear that it is a dynamic biological process that is tightly regulated in time and space. Numerous growth factors, cytokines, enzymes, cellular receptors, ECM components, and signal molecules have been identified as modulators of the angiogenic process and each have their own specific function. Under normal physiological circumstances there is a balance between endogenous angiogenesis inhibitors and stimulators. Disturbance of this equilibrium leads to numerous pathological conditions. Exorbitant and uncontrolled blood vessel growth is implicated in rheumatoid arthritis, psoriasis, and age-related macular degeneration, and it is a hallmark of cancer growth and metastasis. Theoretically, these diseases could be treated by inhibiting angiogenesis. On the other hand, insufficient vessel growth and abnormal vessel regression not only cause heart and brain ischemia, but can also lead to neurodegeneration, atherosclerosis, and chronic wounds [9,19]. The discovery of numerous proangiogenic factors and their promising preclinical use in the 1990s–2000s led to numerous agents that entered clinical trials. These novel angiogenic therapeutic approaches attempted to stimulate vascular growth by protein or gene therapy. In protein therapy, recombinant proteins such as VEGF and fibroblast growth factor-2 (FGF-2) were used to enhance angiogenesis. Gene therapy in this setting involves the delivery of a gene construct of a proangiogenic gene using viral or nonviral vectors to MSCs. Unfortunately, most of these clinical trials to stimulate blood vessel growth had disappointing results and did not meet their goals [2,20]. Potential reasons for these failures include the short half-life of recombinant proteins and, in the case of gene therapy, poor transgene expression [20–22]. Therefore, current scientific focus has shifted from protein and gene therapy to the use of stem cells, and particularly MSCs. The concept of the application of MSCs is that the transplanted cells function as protein factories, continuously producing a cocktail of various endogenous proangiogenic factors and thus inducing angiogenesis via a paracrine mechanism. The proangiogenic properties of MSCs also make these cells an attractive cell source for tissue engineering. Rapid vascularization of newly implanted tissue constructs is an essential prerequisite for the long-term survival and function of these tissues [23].

## 25.3 Proangiogenic properties of mesenchymal stem/stromal cells

### 25.3.1 The mesenchymal stem/stromal cell secretome: a kaleidoscope of angiogenic molecules

Initially, MSCs gained attention in regenerative medicine because of their potential to differentiate into a variety of mesodermal cell types. Despite their long-lasting therapeutic efficacy in numerous *in vivo* preclinical models and clinical trials, the incidence of MSC engraftment in the injured tissue has been found to be surprisingly low. Therefore, a paradigm shift occurred, indicating that paracrine effects rather than transdifferentiation, at least in non-mesodermal tissues, are responsible for the beneficial effects of MSCs [24]. As a consequence, efforts were made to determine whether the conditioned medium (CM) of MSCs rather than MSCs themselves could be used as a therapeutic agent.

Thus, the rationale for using MSCs in diseases in which inadequate angiogenesis plays a key role is based on the fact that MSCs can secrete a number of proangiogenic proteins. With enzyme-linked immunosorbent assay, antibody arrays, and immunohistochemistry a broad repertoire of growth factors, cytokines, and other proangiogenic mediators has been detected in the CM from MSC cultures (as an indicator of the MSC secretome) from a number of MSC tissue sources. Table 25.1 summarizes the most important proangiogenic factors, their exact role in angiogenesis and the MSC type in which they were discovered.

In MSCs isolated from bone marrow (BM-MSCs) various angiogenic molecules were found, including VEGF, FGF-2, Ang-1, MCP-1, MMP-2, MIP-1β, IL-6, and PLGF [25,26,28,34,38–41]. Advanced mass spectrometry has demonstrated the presence of the protein Cyr61 (cysteine-rich angiogenic inducer 61) in the secretome of murine BM-MSCs. An anti-Cyr61 antibody inhibited BM-MSC-induced EC tube formation *in vitro* and blood vessel formation *in vivo*, indicating an important role for Cyr61 in BM-MSC-mediated angiogenesis [27]. Adipose-derived MSCs (AD-MSCs) were shown to produce VEGF, HGF, and TGF-β [30], while high levels of VEGF, MCP-1, and IL-8 were detected in the secretome of human dental pulp stem cells (DPSCs) [36,42]. The production of angiogenic molecules has been shown to be age-dependent and decreases with age in AD-MSCs [43].

Carmeliet and colleagues discovered that not only growth factors and cytokines but also metabolic enzymes and sugars play a key role in blood vessel growth [16–18]. The effect of MSCs on EC metabolism is currently unknown but needs to be explored in order to obtain a detailed insight of the effect of MSCs on angiogenesis.

Communication between MSCs and the cells they interact with, such as ECs, can also be modulated via the production of cell membrane vesicles, which can carry proteins as well as

**Table 25.1** Pro-angiogenic and anti-angiogenic factors.

| Angiogenic factor | Full name | Function | MSC type[a] | Ref. |
|---|---|---|---|---|
| *Pro-angiogenic factor* | | | | |
| Angiogenin | Angiogenin | EC migration, invasion, proliferation, and tube formation | hBM-MSC | [25] |
| Ang-1 | Angiopoietin-1 | Vessel stabilization, EC survival, recruitment of pericytes | mBM-MSC | [26] |
| Ang-2 | Angiopoietin-2 | EC migration and sprouting | mBM-MSC | [26] |
| Cyr61 | Cysteine-rich 61 | EC cell adhesion and EC migration | mBM-MSC | [27] |
| FGF-2 | Fibroblast growth factor-2 | EC proliferation, migration, remodeling of the ECM | mBM-MSC | [28] |
| HGF | Hepatocyte growth factor | EC and smooth muscle cell proliferation, migration | rBM-MSC | [29] |
| | | | hAD-MSC | [30] |
| IGF-1 | Insulin-like-growth factor-1 | EC proliferation, survival, induction VEGF and plasminogen activators | rBM-MSC | [29] |
| IL-6 | Interleukin-6 | EC proliferation and migration | hBM-MSC | [25] |
| | | | hPl-MSC | [31] |
| IL-8 | Interleukin-8 | EC proliferation, survival, migration, and tube formation | hBM-MSC | [32,33] |
| MCP-1 | Monocyte chemoattractant protein-1 | EC migration | mBM-MSC | [28,34] |
| | | | hBM-MSC | [25] |
| MIP-1α | Macrophage inflammatory protein-1α | Proinflammation, chemoattractants of immune cells | mBM-MSC | [34] |
| MIP-1β | Macrophage inflammatory protein-1β | Proinflammation, chemoattractants of immune cells | mBM-MSC | [34] |
| MMP-2 | Matrix metalloprotease 2 | ECM degradation, cell migration | hBM-MSC | [35] |
| NAP-2 (CXCL7) | Neutrophil-activating protein 2 | Neutrophil recruitment, EC migration, release of VEGF and MMP | hBM-MSC | [25] |
| PLGF | Placental growth factor | Induction of vessel formation | mBM-MSC | [28] |
| TGF-β | Tumor growth factor-β | Tube formation, vessel stabilization, ECM synthesis | hAD-MSC | [30] |
| VEGF | Vascular endothelial growth factor | Increased vessel permeability, ECM degradation, EC proliferation, migration, tube formation and survival | mBM-MSC | [26,28] |
| | | | hBM-MSC | [25] |
| | | | hDPSC | [36,37] |
| | | | hAD-MSC | [30] |
| *Anti-angiogenic factor* | | | | |
| TIMP-1 | Tissue inhibitor of metalloproteinase-1 | Inhibitor of metalloproteinases | hBM-MSC | [25] |
| TIMP-2 | Tissue inhibitor of metalloproteinase-2, | Inhibitor of metalloproteinases | hBM-MSC | [25] |
| MIG | Monokine induced by interferon-γ | Chemoattractant of T-cells, inhibition of angiogenesis | mBM-MSC | [34] |

[a] h: human; m: mouse; r: rat.

genetic information via microRNAs (miRNAs) (see Chapters 20 and 37). The miRNAs are able to target paracrine factors and molecular pathways primarily through posttranscriptional repression or mRNA degradation in a sequence-specific manner. Growing evidence indicates that various miRNAs are involved in various steps of the angiogenic process, including proliferation and migration of ECs [44]. For example, miR-210 is able to promote angiogenesis in ECs by downregulating ephrin-A3 [45], while miR-126 has been shown to upregulate VEGF and to participate in EC sprouting in response to blood flow [46]. The miRNA profile of normal and hypoxia-treated rat BM-MSCs were compared and 13 miRNAs were significantly upregulated (including miR-210), whereas 20 miRNAs (such as miR-377) were downregulated. miR-377 was shown to negatively regulate the expression of VEGF; as a consequence, MSCs in which miR-377 was knocked down promoted angiogenesis in a preclinical model of myocardial infarction [47]. The miRNA network involved in the angiogenic actions of MSCs remains to be fully

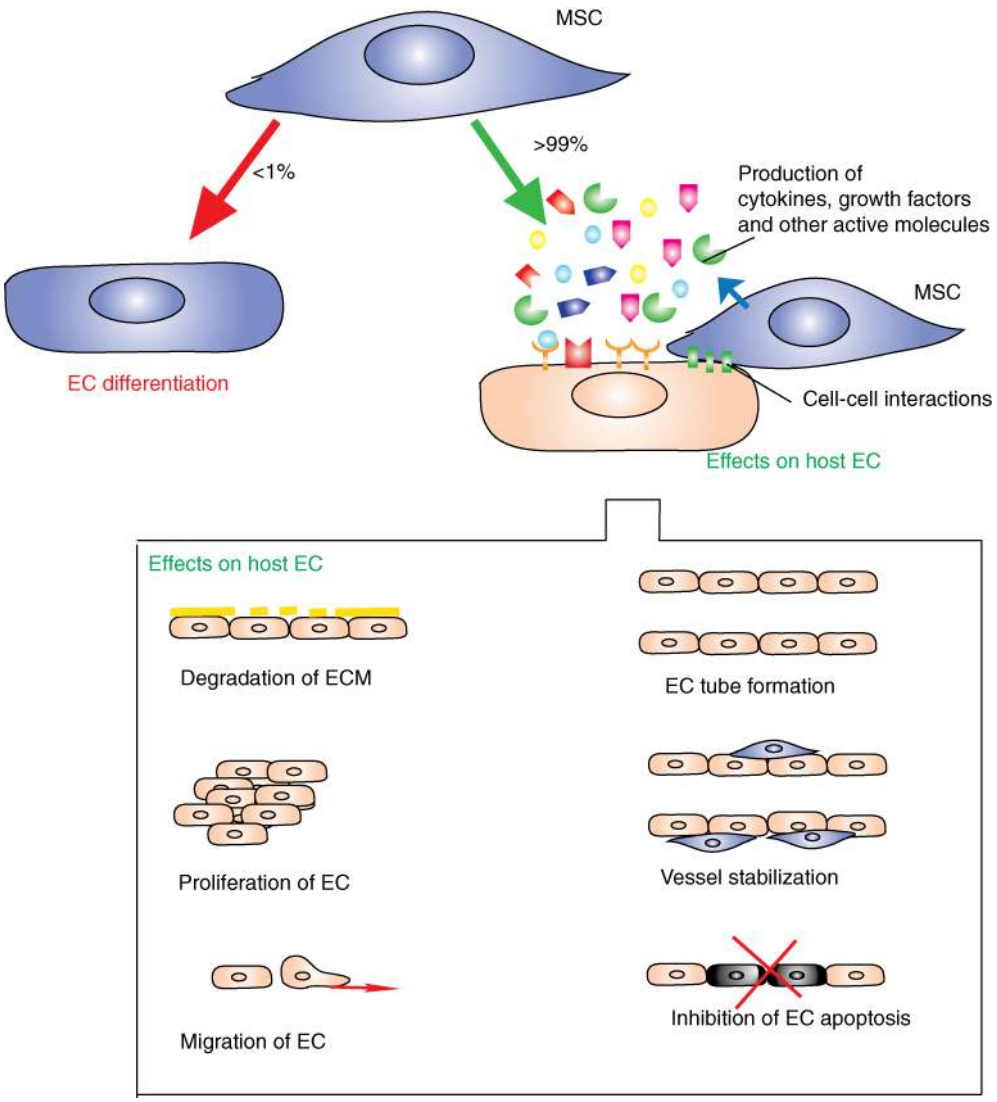



**Figure 25.3** Angiogenic actions of MSCs. Incorporation of MSCs into the host vasculature is rarely observed, the current paradigm being that MSCs induce angiogenesis mostly by activating host ECs by cell–cell contact or by secreting various angiogenic molecules. MSCs are involved in various steps of the angiogenic process, such as breakdown of the ECM, EC proliferation, migration, tube formation, and vessel stabilization. They can also protect ECs from apoptosis.

elucidated. Insight of the miRNA network responsible for the regulation of MSC-induced angiogenesis may lead to new therapeutic approaches [44].

### 25.3.2 The effect of mesenchymal stem/stromal cells on the behavior of endodothelial cells *in vitro*

In order to assess whether a particular cell population possesses angiogenic activity, it is not only necessary to identify the angiogenic mediators present in their secretome, but also to explore the effect of these cells on the behavior of ECs, the most important factors in neovascularization. EC proliferation, migration, and tube formation are key steps of angiogenesis and there are various *in vitro* models available that mimic each of these phases. Figure 25.3 summarizes the possible contribution of MSCs to each of these processes.

#### 25.3.2.1 Stimulation of endothelial cell proliferation

There is disparity in the literature with regard to the effect of MSCs on EC proliferation. On the one hand, the CM of BM-MSCs has been repeatedly reported to induce EC proliferation [28,40]. In addition, Lin *et al.* demonstrated that CM derived from $CD45^{-}/PDGFR^{+}$ murine MSCs isolated from four different tissue types (bone marrow, adipose tissue, skeletal muscle, and myocardium) stimulated proliferation of human umbilical-vein-derived ECs (HUVECs) [48] (PDFGR is platelet-derived growth factor receptor). Also, the CM of Wharton's jelly and AD-MSCs significantly increased EC growth [30,49].

On the other hand, it been demonstrated using a radioactive-labeled thymidine incorporation assay, that the CM of BM-MSCs did not induce EC growth [50]. This was confirmed by Barlacu *et al.*, who demonstrated no effect of CM from murine BM-MSCs on the proliferation of HUVECs [51]. No effect on EC proliferation was found when the CM from dental MSCs was added to the human EC line HMEC-1 [52].

Some investigators have determined that MSCs inhibit EC proliferation. For example, co-culture of ECs with MSCs on poly (L-lactide-co-1,5-dioxepan-2-one) scaffolds in a dynamic culture environment resulted in lower values of the cell proliferation markers proliferating cell nuclear antigen and Ki67, and thus proliferating ECs [53]. Menge *et al.* reported that 24 h of co-culture of MSCs with ECs resulted in reduced proliferation and a decreasing number of ECs in the S phase of the cell cycle. A transwell culture of MSCs with ECs, which does not permit direct cell–cell contact between MSCs and ECs, had no effect on the amount of ECs in S phase, leading to the conclusion that EC–MSC cell-to-cell contact is required [54]. BM-MSC-derived exosomes also inhibited proliferation of the murine EC line SVEC *in vitro* [55]. These inconsistent findings in the literature may be due to the heterogeneity of the MSCs, different sources of ECs, differences in assays to monitor EC proliferation, variations in preparation of the CM used (e.g., serum concentrations, basal medium used), and the use of CM versus co-culture with cells.

#### 25.3.2.2 Induction of endothelial cell migration

Numerous reports have shown that MSCs induce *in vitro* transwell migration of ECs [36,48–51,56]. In 2005 it was demonstrated that CM from BM-MSC cultures was chemotactic for both human umbilical vascular and uterine microvascular ECs [50]: Gruber *et al.* reported that addition of anti-VEGF antibodies had no effect in this assay, while Kwon *et al.* [40] described significant inhibition with a single neutralizing antibody against VEGF, MCP-1, or IL-6 but not by antibodies against TGF-β1 and HGF. MSCs derived from pulp were able to induce EC migration in a transwell assay. The migration-inducing capacities of these DPSCs was inhibited by an antibody against VEGF or by the PI3K-inhibitor LY294002 (10 and 1 μM) and the mitogen/extracellular signal-regulated kinase (MEK) inhibitor U0126 (10 and 1 μM). In addition, CM from MSCs derived from adipose tissue, skeletal muscle, and myocardium induced motility of ECs in the *in vitro* scratch assay [48]. The *in vitro* scratch assay is an easy and well-developed method to measure cell migration *in vitro*. The basic steps involve creating a "scratch" in a cell monolayer, capturing the images at the beginning and at regular intervals during cell migration to close the scratch, and comparing the images to quantify the migration rate of the cells.

#### 25.3.2.3 Stimulation of tube formation

The ability of MSCs to induce tube formation of ECs plated on an ECM component such as Matrigel is one of the best described measurements of *in vitro* angiogenesis. The CM of BM-MSCs is able to stimulate network formation of various ECs *in vitro* on collagen or Matrigel [27,34,40,50,56]. Depletion of Cyr61, VEGF, MCP-1, and IL-6 but not HGF and TGF-β1 significantly attenuated this MSC-mediated tube formation [27,40]. Ghajar *et al.* observed that co-culture of human BM-MSCs with HUVECs induced formation of a denser network in a three-dimensional fibrin gel [57]. Human MSCs derived from Wharton's jelly, dental pulp, adipose tissue, skeletal muscle, and myocardium stimulate EC tube formation [48,49,52].

#### 25.3.2.4 Enhancment of matrix degradation

The ability of ECs to degrade the surrounding ECM *in vitro* in the cell invasion assay is stimulated by MSCs. The cell invasion assay monitors cell movement through ECMs. AD-MSCs as well as BM-MSCs have been shown to induce EC invasion, through upregulation of distinct proteases [28,58]. In a three-dimensional model of network formation in fibrin gel, addition of human MSCs resulted in a denser network formation of HUVECs. Induction of early production of MMPs, including the soluble forms of MMP-2 and MMP-9 as well as membrane-bound MT1-MMP was shown to be the underlying mechanism, suggesting that MSCs contribute to angiogenesis by controlling extracellular proteolytic mechanisms [57]. Annabi *et al.* demonstrated that murine BM-MSCs were able to produce active MMP-2, which was able to degrade the ECM [32].

#### 25.3.2.5 Blood vessel stabilization and maturation

The origin of MSCs has been the subject of considerable discussion, but currently it is assumed that at least a significant population of MSCs in most tissues are derived from perivascular cells [24,59]. Therefore, it is not surprising that data indicate that both *in vitro* and *in vivo* MSCs play a role in tube stabilization. It was demonstrated that human BM-MSCs in direct co-culture with ECs on Matrigel increased the persistence of tubular structures compared with culture of ECs alone. This effect was considered to be caused by direct cell-to-cell contact between the BM-MSCs and ECs [60]. In a three-dimensional Matrigel co-culture system BM-MSCs co-aligned with tubular EC structures in a pericyte-like manner and some BM-MSC populations enhanced the complexity and extent of these tubes [61]. A recent study demonstrated that co-culture of MSCs with ECs on poly(L-lactide-co-1,5-dioxepan-2-one) scaffolds in a dynamic culture environment resulted in a quiescence of the ECs, as described earlier. The ECs displayed lower proliferation and increased secretion of markers of vascular maturation such as angiopoietin-1 [53]. In another study, human BM-MSCs efficiently stabilized nascent blood vessels by functioning as perivascular precursor cells in an *in vivo* tissue-engineered blood vessel construct [62]. In the light of these findings, it is worth mentioning that *in vitro* BM-MSCs have the ability to adhere to human ECs from arteries, veins, and microvasculature under static conditions. When injected into the chick embryo chorioallantoic membrane (CAM), human MSCs adhered to arterioles. Inhibition of α4 integrin and/or sialyl Lewis X with blocking antibodies reduced adhesion in arterioles [63]. Ball *et al.* demonstrated that

the receptor neuropilin-1 (NRP1) co-localizes with PDGFR and is also involved in the EC adherence and blood-vessel-stabilizing function of MSCs [64]. NRP1 is a membrane-bound co-receptor to a tyrosine kinase receptor for both VEGF and semaphorin family members.

Otsu *et al.* reported that addition of MSCs to EC-derived tubes in a two-dimensional *in vitro* Matrigel assay led to degeneration of the network. MSCs were shown to intercalate between the ECs, formed gap junctions with the ECs, and caused production of reactive oxygen species that resulted in EC apoptosis and destruction of the tubes. It should be noted that these cytotoxic effects of MSCs were only evident when the MSCs were added to the Matrigel in very high numbers with an EC : MSC ratio of 1 : 1 or 1 : 3. The toxic effects were dramatically reduced when the number of MSCs was decreased [65]. This dose dependency might also explain the difference between the findings of Otsu *et al.* and the overwhelming evidence of the proangiogenic effects of MSCs. Menge *et al.* showed that ECs which have been co-cultured with MSCs form a smaller network on Matrigel in comparison with control ECs. Interestingly, these effects appear to require EC–MSC contact and result in enhanced co-localization of VE-cadherin and β-catenin on the EC membrane. ECs cultured in contact with Wnt3a-overexpressing MSCs regained the capability of forming EC networks in Matrigel [54].

#### 25.3.2.6 Protection of endothelial cells against apoptosis

MSCs also have the capacity to protect ECs from apoptosis *in vitro*. CM of hypoxic AD-MSCs inhibited EC death induced by cycloheximide and TNF-α [30]. In addition CM of BM-MSCs reduced hypoxia-induced apoptosis of ECs through activation of the PI3K/Akt pathway but not the ERK pathway [25]. (ERKs are extracellular-signal-regulated kinases and are now known as mitogen-activated protein kinases (MAPKs); they are widely expressed protein kinase intracellular signaling molecules that are involved in functions including the regulation of meiosis, mitosis, and postmitotic functions in differentiated cells.) Many different stimuli including growth factors, cytokines, virus infection, ligands for heterotrimeric G protein-coupled receptors, transforming agents, and carcinogens activate the ERK pathway. The term "extracellular-signal-regulated kinases" is sometimes used as a synonym for MAPK, but has more recently been adopted for a specific subset of the mammalian MAPK family. Placental MSCs abrogated oxidative stress-provoked apoptosis induced by *tert*-butyl hydroperoxide via the signal transducer and activator of transcription 3 (STAT3) signal transduction cascade [31]. (*tert*-Butyl hydroperoxide is an organic peroxide widely used in a variety of oxidation processes.)

### 25.3.3 Mesenchymal stem/stromal cells induce angiogenesis *in vivo*

There is little doubt that the angiogenic effect of molecules or cells is best evaluated *in vivo*. In the field of embryology the two most commonly used animal models to study blood vessel formation in absence of any pathology are the chicken CAM assay and the mouse Matrigel plug assay. In this section we discuss the effect of MSCs on angiogenesis in each of these *in vivo* models.

#### 25.3.3.1 The chicken chorioallantoic membrane assay

Since the adaptation of this animal model by Judah Folkman in the early 1970s the CAM assay has been widely used to investigate pro- and anti-angiogenic agents. The CAM is an extraembryonic highly vascularized membrane that serves as a gas exchange surface for the embryonic chick until hatching. One of the advantages of this model is that at this developmental stage the immune system is not fully developed and the mechanisms for rejection of applied agents have not been established. As this model represents a simple and cost-effective procedure with limited ethical concerns, it has become a practical and intensively used research tool [66]. Traditionally, it has been applied to study both angiogenesis and anti-angiogenesis in response to chemical molecules, proteins, cancer cells, and tumor biopsy specimens. Recently, the model has gained attention in the field of tissue engineering to assess neoangiogenesis induced by cells or engineered cellular scaffolds [23].

The CM of murine BM-MSCs and especially that of murine BM-MSCs pretreated with TNF-α- and/or interferon-γ-induced blood vessel formation in the CAM [50,67]. Additionally, human BM-MSCs stimulated blood vessel formation in this model. This angiogenic effect was even more profound with BM-MSCs that had been cultured in serum-deprived conditions for 30 days [68]. DPSCs and placenta-derived MSCs also induced capillary formation in this model [52,69,70]. CAMs transplanted with endothelially differentiated placental MSCs stimulated a greater number of blood vessels compared with CAMs transplanted with undifferentiated human placenta-derived MSCs. Function-blocking antibodies to integrins $\alpha_5$ and $\beta_1$ prevented this human placenta-derived MSC-mediated blood vessel induction [69].

#### 25.3.3.2 The murine Matrigel plug assay

The mouse Matrigel plug assay is a relatively easy *in vivo* technique with which to validate angiogenesis. Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth–Holm–Swarm (EHS) mouse sarcoma cells and is used as a basement membrane preparation in laboratory research. The exact composition of Matrigel is unknown, but it contains ECM proteins such as laminin, collagen IV, and entactin. Usually, a growth factor, anti-angiogenic agent, or cells are mixed with liquid Matrigel and injected subcutaneously in immunocompromised mice. At the body temperature of the mouse the Matrigel mixture solidifies and forms a plug. This plug can be recovered 7–21 days later to examine the number of blood vessels that have entered it. Quantification of blood vessels is usually performed on histological sections, which is tedious but accurate. Additionally, the hemoglobin content of the plug can be measured as a measure of angiogenesis, but this can be misleading as blood content can also be affected by blood vessel size or by stagnant pools of blood [1].

Most studies indicate that Matrigel plugs seeded with BM-MSCs have an increased blood vessel content compared with control Matrigel inserts [27,71]. Verfaillie's group found that BM-MSCs are able to attract host blood vessels to subcutaneous Matrigel transplants measured 3 weeks after injection, but that these tubes are leaky, as evidenced by the presence of red blood cell pools in the Matrigel [72]. A study by Janebodin *et al.* indicated that DPSC-containing Matrigels had more blood vessels then BM-MSCs plugs, and this neovascularization was reduced by adding soluble VEGF-receptor 1 [73]. In contrast, Menge *et al.* reported that when MSCs were injected via the tail vein they inhibited blood vessel growth in Matrigel plugs seeded with FGF-2 or MSCs. It was hypothesized that this anti-angiogenic effect of the MSCs was caused by increased IL-10 production of MSCs residing in the lung tissue after intravenous administration, but further investigation is needed [54].

## 25.4 Mesenchymal stem/stromal cells as a therapeutic tool for diseases caused by insufficient angiogenesis

In this section we discuss the effects of MSC administration in animal models of a wide variety of disorders caused by limited angiogenesis. Key observations that exemplify the pro-angiogenic impact of MSC administration will be highlighted. We will also discuss human clinical trials with MSCs in these disorders.

### 25.4.1 Peripheral ischemic arterial disease

Peripheral ischemic arterial disease is a narrowing or obstruction of the arteries of the limbs resulting from pathological changes in the arteries, such as atherosclerosis or inflammation. This results in a wide variety of symptoms and signs, ranging from weakness and cramping in the muscles to chronic wounds, ulcers, and in later stages muscle atrophy and gangrene. Since there are currently no pharmacological treatments for this, amputation of the affected limb is required in one-third of cases if the arterial blockage cannot be reversed [74]. Because of their angiogenic potential, MSCs may help to restore the blood flow to tissues distal to the obstruction. Peripheral ischemic arterial disease is mimicked in animal models by blocking the blood flow by temporary ligation or resection of important hind limb arteries such as the femoral artery, resulting in ischemia of the leg [75].

In 2004, Kinnaird *et al.* showed in a mouse model of hindlimb ischemia that the local intramuscular injection of BM-MSCs increased blood vessel density and limb perfusion, reduced the incidence of autoamputation, attenuated muscle atrophy, and significantly improved limb function. No MSC incorporation into the blood vessel walls was observed, but both VEGF and FGF-2 levels were elevated in the BM-MSC-treated muscles, suggesting that paracrine actions of MSCs were responsible for the amelioration of the BM-MSC-treated group [28]. In contrast, Iwase *et al.* and Al-Khaldi *et al.* found evidence in rat models of hindlimb ischemia of transplanted BM-MSCs in recipient blood vessel walls expressing EC markers such as von Willebrand factor (vWF) and CD34 at 3 or 4 weeks after administration. However, neither study quantitated the number of engrafted MSCs [75,76]. In both studies the BM-MSC-treated rats displayed increased limb perfusion. Intravenous injection of human AD-MSCs improved blood perfusion in a mouse hindlimb ischemia model [30,77]. Implanted DiI-labeled human AD-MSCs also seemed to differentiate into ECs as vWF/DiI double-positive cells were detected within the muscle tissue. The actual incorporation rate of these cells into the recipient's vascular structures was less than 1%, again suggesting that paracrine effects of AD-MSCs accounted for the beneficial effect on hindlimb ischemia [77]. In addition, the intramuscular injection of a side population of $CD31^-$ $CD146^-$ porcine DPSCs increased blood flow and capillary density in a murine hindlimb ischemia model. Transplanted cells were located in close vicinity to the blood capillaries. *In situ* hybridization revealed the expression of VEGF and MMP-3 by these engrafted porcine DPSC side population cells [78].

A phase II clinical trial demonstrated that the intra-arterial infusion of autologous bone marrow significantly increased both peripheral blood perfusion and the percentage of amputation-free patients with critical limb ischemia 1 year after the infusion [79].

### 25.4.2 Stroke

Stroke, also known as acute cerebral ischemia, is a sudden neurologic deficit caused by a disturbance in the blood supply to the brain. The most frequent cause of stroke (80–85%) is thromboembolism or an *in situ* thrombosis, blocking the blood circulation and leading to a decrease in oxygen and nutrient delivery to the brain. Cerebral hemorrhage resulting from hypertension or arterial wall pathology can also lead to stroke [80]. Depending on the affected area of the brain, ischemic stroke is associated with temporary or permanent loss of brain tissue and function, resulting in severe motor, sensory, and cognitive dysfunction. Cerebral ischemia or stroke is the most common cause of adult-acquired disability and the second leading cause of death worldwide [81]. Currently, the only pharmacological treatment available is tissue plasminogen activator for lysis of the blood clot in thrombotic stroke and its use is limited to a small minority of patients because of the narrow therapeutic window of 4.5 h after disruption of blood flow. It is contra-indicated in hemorrhagic stroke.

The hypothesis that MSCs could transdifferentiate into neural cells was the first rationale for their use in stroke. MSC-derived "neuronal" cells may look like neurons with long dendritic processes and may even stain positively for antigens normally restricted to neural cells such as β3-tubulin. However, functionally, they do not fire action potentials [82,83] and this transdifferentiation to neural cells has not been reproduced *in vivo* [84]. Moreover, it has been observed that MSCs only transiently engraft in the ischemic brain. A study in which human MSCs were injected intracranially after stroke induction in mice found no administered cells detectable on histological

sections 28 days later [85]. Despite this low rate of engraftement in the recipient, numerous reports have shown that BM-MSCs improve neurological function in several animal models of stroke. This is most likely mediated through a combination of protection against apoptosis, immunomodulation, stimulation of neural plasticity, and induction of angiogenesis [63,86]. These favorable effects of MSCs on functional recovery have been found to be robust across rodent and primate species, route of delivery, and type and source of MSCs [87]. Numerous studies have indicated that the improved recovery after MSC administration is associated with an increase in blood vessel density and production of angiogenic factors. For example, increased VEGF and VEGF-receptor (VEGFR)2 expression was observed after intravenous injection of human BM-MSCs in a rat model of ischemic stroke [88].

Another research group reported a significant upregulation of Ang-1 and Ang-2 mRNA levels in BM-MSC-treated rats [89]. The intraperitoneal injection of a cell-free extract derived from MSCs was shown to dramatically decrease the ischemic volume and improve motor function after stroke injury [90].

This extensive work on MSC administration in animal models of stroke injury has encouraged the study of these cells in phase I/II clinical trials [86]. Bang *et al.* were the first to describe a trial using autologous BM-MSCs: five patients received autologous BM-MSCs intravenously. Although the BM-MSC-treated group had a tendency toward a better functional outcome, this difference was not statistically significant [91]. Subsequently, the same research group published the results of a long-term follow-up study of the intravenous administration of autologous BM-MSC transplantation in patients with stroke injury that showed BM-MSC treatment improved functional outcome and survival for up to 5 years after MSC administration [92]. A nonblinded trial of 12 patients given autologous BM-MSCs expanded *ex vivo* using autologous serum indicated that the intravenous administration of these cells 36–133 days after the stroke was not only safe and feasible but could also reduce infarct size by 20% at 1 week after infusion [92]. All these clinical trials had a small sample size and the study by Honmou *et al.* [93] was nonblinded; therefore, a placebo effect could not be excluded. However, the results were encouraging enough to warrant randomized placebo-controlled double-blinded trials (see Chapter 48).

### 25.4.3 Myocardial infarction

Cardiovascular disease is responsible for >45% of all deaths in western countries and is reaching pandemic proportions worldwide. Acute myocardial infarction (AMI) resulting from ischemic coronary artery disease represents a major economic burden on the health-care system of many countries since many survivors of the acute ischemic episode subsequently develop chronic myocardial ischemia and complications such as congestive heart failure, which is often refractory to current conventional treatment [94]. The administration of MSCs is currently being explored as a potential therapeutic agent for both and chronic ischemic myocardial disease (see Chapter 45). Initial preclinical animal studies and early clinical trials have shown encouraging results. Initially, it was assumed that MSCs might improve cardiac function after AMI due to their transdifferentiation into cardiomyocytes. However, significant *in vivo* MSC engraftment and cardiomyocyte differentiation was found to be nonexistent or very rare [94–98] and this gave rise to the hypothesis that MSCs repair the damaged heart by supplying growth factors that induce angiogenesis or by stimulating resident cardiac stem cell migration and differentiation [94,99]. Numerous preclinical studies have demonstrated the functional effectiveness of MSCs from a number of different tissue sources, including bone marrow, adipose tissue, dental pulp, and amnion [96,97,100,101]. Administration of these cells resulted in more normal myocardial remodeling rather than the pathological remodeling with extensive myocardial fibrosis that is often a result of AMI, reduced infarct size, reduced myocardial fibrosis, improved cardiac contractile function, and stimulation of angiogenesis. Secretion of both VEGF and HGF was found to contribute to improved cardiac function after transplantation with AD-MSCs [100]. The critical role of VEGF in MSC-induced blood vessel growth after myocardial infarction was also demonstrated by Markel *et al.*, who described that small interfering RNA (siRNA) directed against VEGF inhibited the recovery of myocardial function in a rat model of AMI [102]. In addition, the intramyocardial injection of amniotic MSCs not only increased the levels of VEGF but also of Ang-1 in the affected heart tissue [101]. In a hamster model of cardiomyopathy, injection of porcine BM-MSCs into the hamstring skeletal muscles improved cardiac repair due to their production of multiple IL-6-like cytokines [103].

The safety, dose, and impact associated with cell administration for myocardial infarction in humans have been extensively studied and published [94,99,104]. Several trials, such as the BOOST trial (BOne marrOw transfer to enhance ST-elevation infarct regeneration) have shown an increase in left ventricular ejection fraction in recipients of autologous bone marrow cells compared with control groups at 4, 12, and 18 months after cell administration [105–107]. However, the exact type of bone marrow cells infused in the BOOST trial is unclear since the cells were infused after only an average of 5 days of culture.

Measurement of left ventricular ejection fraction is the commonest parameter for evaluating function of the left ventricle. AMIs may be ST segment elevation myocardial infarctions (STEMI) or non-STEMI myocardial infarctions with regard to the presence or absence of elevation of the ST waves on the electrocardiogram: STEMI is a more serious type of AMI than non-STEMI.

Three other clinical trials demonstrated no significant improvement in cardiac function after injection of bone marrow mononuclear or progenitor cells [108–110]. Again, it should be emphasized that the injected cells were not MSCs that had been expanded in long-term *ex vivo* culture. At 5 years after treatment there are discrepant reports in the literature: a 5-year follow-up of the patients enrolled in the BOOST trial showed that the beneficial effects observed at 18 months in terms of cardiac

contractility were not sustained, while another 5-year follow-up study did demonstrate a significant improvement in left ventricular ejection fraction in the bone-marrow-cell-treated AMI patients [111,112].

It is now generally agreed that bone marrow mononuclear cells and hematopoietic progenitor or hematopoietic stem cells are of limited use for the treatment of acute and chronic myocardial ischemia.

The focus on cellular treatment for ischemic heart disease has now turned to the use of MSCs (see Chapter 45). Two small trials demonstrated the safety and potential efficacy of autologous adipose tissue-derived MSC administration [113,114]. There have been, and are, a number of clinical trials using MSCs, including the REVITALIZE trial (NCT00874354) in which autologous BM-MSCs were injected directly into the myocardium via a catheter inserted in the recipient's femoral artery under local anesthesia. Two phase II clinical trials have been initiated using AD-MSCs [94,115]. Full details of the use of MSCs for both acute and chronic ischemic heart disease are given in Chapter 45.

### 25.4.4 Failure of surface wound healing

Surface wound healing is a complex multifactorial process involving the interaction of inflammation, re-epithelialization, angiogenesis, and remodeling of the ECM. In pathological settings such as severe burns or diabetes mellitus [116,117] normal wound repair mechanisms fail to restore skin function, leading to treatment-refractory skin ulceration. Current biological techniques used for the healing of severe wounds include the use of antimicrobial support combined with a matrix scaffold that in most cases is collagen-based and seeded with growth factors.

However, outcomes in this field remain marginal and therefore MSC-based applications are being explored [116,117]. Both allogeneic and autologous MSCs administered either locally or systemically accelerate wound closure in a wide variety of animal models [118]. In a study by Wu *et al.* BM-MSC treatment of wounds in diabetic mice resulted in accelerated wound healing, increased re-epithelialization and wound vascularity probably through the release of Ang-1 and VEGF [26]. In this study, the rate of MSC engraftment 28 days after injection was extremely low at less than 1% of the MSCs administered. No integration of the transplanted $GFP^+$ BM-MSCs into the recipients' vasculature was observed [26]. Application of AD-MSCs in cell sheets consisting of poly-*N*-isopropylacrylamide accelerated wound closure and angiogenesis in nude mice [119]. Also, concentrated CM of BM-MSCs and CM of hypoxia-pretreated AD-MSCs were both reported to enhance wound healing in a murine model of excisional wounds [120,121]. Addition of neutralizing antibodies against VEGF and FGF-2 to the wounds treated with the AD-MSC CM reduced the wound repair, indicating that VEGF and FGF-2 are key molecules in wound healing [121]. Another study considered the beneficial effect of the BM-MSC CM to be due to the recruitment of macrophages and endothelial progenitor cells to the site of injury [120]. Another study claimed that besides induction of angiogenesis, MSCs could contribute to wound repair by differentiation into keratinocytes [122], although evidence for such transdifferentiation has not been provided in all studies [118].

Initial clinical studies indicate a possible therapeutic role for MSCs in wound healing. A small pilot study in patients with acute or severe chronic wounds showed that the topical administration of autologous BM-MSCs in a fibrin spray significantly reduced wound size [123]. Another trial in which autologous BM-MSCs were administered to people with chronic nonhealing ulcers of the lower extremities showed that the MSC-treated group had significant reduction in ulcer size and improvement in pain-free walking distance compared with the control group [124]. Another study found both bone marrow mononuclear cells and tissue culture-expanded $CD90^+$ cells useful for the treatment of chronic foot ulcers in diabetic patients: wound healing and improved microvascularization were detected in some but not in all patients in this study [125]. Thus, while the results of preclinical studies and early clinical use of MSCs in the wound healing setting have given rise to optimism, further phase I and II studies are needed to determine the value of MSCs in this pathological setting in humans [116] (see Chapters 58 and 59).

### 25.4.5 The dual role of mesenchymal stem/stromal cells in cancer biology

As MSCs are able to induce blood vessel formation and since tumor growth and metastases are angiogenesis dependent, the contribution of MSCs to cancer development has been the subject of intense investigation. During tumor growth endogenous MSCs are recruited from the bone marrow to the tumor where they become a component of the tumor stroma, differentiating into tumor-associated fibroblasts and pericytes. These tumor-associated MSCs have been found in both animal models and clinical settings, and most data indicate that MSCs enhance tumor growth and metastasis [126,127]. However, in other animal studies in which MSCs were added exogenously by local or systemic injection the impact of MSCs on tumor progression was unclear.

The majority of the studies that demonstrated promotion of tumor growth by MSCs were in various cancer models, including lung adenocarcinoma [128], melanoma [129], and breast cancer [130] and are reviewed in References [126] and [127]. Although the molecular mechanisms behind the tumor-promoting capacities of MSCs are still not fully understood, several papers indicate that stimulation of angiogenesis is a crucial element [31,50,52,55,131]. In a mouse xenograft model of colorectal carcinoma it was demonstrated that BM-MSCs increased tumor growth by secreting IL-6, thereby inducing endothelin-1 expression by the cancer cells and activating ECs and thus blood vessel development. siRNA against IL-6 inhibited the MSC-induced tumor development [132]. Liu *et al.* demonstrated that inhibition of VEGF or HIF-1α with siRNA effectively reduced MSC promotion of colon tumor growth *in vivo* [67].

It has been reported that pretreatment of the gastric tumor cell line SGC-7901 with a single dose of human BM-MSC-derived CM *in vitro* was sufficient to augment tumor growth. This human MSC CM induced expression of VEGF by the tumor cells as well as the activation of RhoA-GTPase and ERK1/2 pathways [133]. Besides augmentation of angiogenesis, other suggested mechanisms of tumor growth stimulation include immunomodulation, induction of vasculogenesis, and reduction of tumor cell apoptosis [127].

In contrast to these studies, Khakoo *et al.* showed in a mouse model of Kaposi's sarcoma that human MSCs injected intravenously potently inhibited tumor growth, probably due to inhibition of Akt activity [134]. Otsu *et al.* reported that rat BM-MSCs inhibited the growth of mouse melanoma cells by inhibiting angiogenesis. It is likely that the high number of added cells ($10^6$) and the direct injection of the cells into the tumor caused the development of reactive oxygen species with consequent cytotoxicity [65]. Co-injections of other MSC types such as AD-MSCs, umbilical-cord-blood-derived MSCs, and fetal skin MSCs were shown to reduce tumor size in animal models of glioma, hepatoma, and pancreatic carcinoma [135–137]. Exosomes derived from murine BM-MSCs decreased tumor volume and suppressed angiogenesis by downregulating VEGF expression in a mouse model of breast cancer [55]. The tumor-suppressing capacities of MSCs have been attributed to the induction of apoptosis and inflammation, inhibition of angiogenesis, and alteration of the cell cycle progression [127].

The paradoxical literature findings may be due to variations in MSC source, the heterogenic nature of MSCs, the protocols used in their isolation and culture, patient variability, tumor cell types, contamination of the MSCs with tumor cells, and differences in timing, dose, and mode of MSC administration. Most investigators that reported tumor-promoting effects of MSCs mixed MSCs with tumor cells and injected them simultaneously. The presence of MSCs during the early phases of tumor development may rapidly induce angiogenesis, which in turn could result in substantial tumor growth [127] (and see Chapter 28).

Despite the lack of clarity on the exact involvement of MSCs in tumor pathogenesis, MSCs are becoming increasingly used as delivery vehicles of anticancer gene therapy and chemotherapy (see Chapters 60 and 62). MSCs have the intriguing property of homing to tumors just as they do to sites of nonmalignant inflammation. This preferential migration has been convincingly demonstrated in a number of xenograft models, including those involving hepatocellular carcinoma, breast cancer, and ovarian carcinoma [138–140]. MSCs do not only selectively home to the primary site of solid tumors, but also to metastases far removed from the primary site [141,142]. Their use as cellular vehicles for delivery of genes to tumors includes BM-MSCs overexpressing the suicide gene, herpes simplex virus thymidine kinase (HSV-TK), which successfully reduced tumor volume in a rat glioma model [11]. Should such cells cause side effects in normal tissues, they can be readily eliminated by administration of ganciclovir, which will kill all cells expressing the viral HSV-TK gene but not those cells without it. Another study demonstrated that administration of BM-MSCs overexpressing anti-angiogenic repeats of thrombospondin significantly decreased tumor vessel density and consequently tumor progression in mice with human glioma xenografts [143].

MSC administration used without gene manipulation appears to be safe in humans. A systematic review and meta-analysis of clinical trials showed no serious adverse events associated with the intravenous or intra-arterial administration of MSCs in over 1000 patients, over 300 of whom had received the MSCs in randomized clinical trials. Specifically, there were no *de novo* malignancies reported [144]. The use of MSCs as gene delivery vehicles is an exciting concept but one that will add a significant extra layer of complexity and thus increased regulatory control of their manufacturing for clinical trial purposes.

## 25.5 Enhancing the angiogenic efficacy of mesenchymal stem/stromal cells

Although MSCs display substantial endogenous angiogenic potential, large efforts have been made to further enhance their angiogenic actions [145]. The release of angiogenic factors by MSCs can be significantly augmented by the addition of various chemokines in a process referred to as priming or pharmacological preconditioning or by subjecting MSCs to hypoxia or to serum-free conditions. It has been demonstrated that TGF-α induces the secretion of VEGF, granulocyte colony-stimulating factor, HGF, IL-6, IL-8, the PDGF-BB form of PDGF, and Ang-2 in BM-MSCs. CM from TGF-α-treated MSCs induced more blood vessel growth than CM of untreated BM-MSCs in the *in vivo* CAM assay [33]. Exposure of BM-MSCs to hypoxia, TNF-α, or lipopolysaccharide (LPS) enhanced the release of VEGF, FGF-2, HGF, and insulin-like growth factor 1 (IGF-1) by induction of the transcription factor nuclear factor kappa light chain-enhancer of activated B [146]. Annabi *et al.* demonstrated in murine BM-MSCs that hypoxic conditions downregulated the mRNA and protein levels of MMP2 while those of MT1-MMP were strongly induced [41]. LPS was also able to boost the release of several chemokines, including IL-6 and TNF-α in AD-MSCs. Hypoxia has been shown to elevate the secretion of VEGF, IGF-1, SDF-1, MIP-1α, MIP-1β, and erythropoietin in BM-MSCs, and VEGF, but not FGF-2, secretion by human DPSCs [30,120]. In AD-MSCs, hypoxia selectively increased VEGF, but not FGF, HGF, or TGF-β, mRNA and protein levels in AD-MSCs [30].

Oskowitz *et al.* showed that serum deprivation for 30 days induced the release of VEGF, HGF, IGF-1, and angiogenin by human BM-MSCs. CM of these serum-deprived cells generated longer neovascular sprouts in the *ex vivo* aortic ring assay compared with CM of control BM-MSCs [68].

## 25.6 Transdifferentiation of mesenchymal stem/stromal cells towards endothelial cells

The rate of *in vivo* engraftment of MSCs within the blood vessels remains very low in most settings. This is the basis of the assumption that the angiogenic effect of MSCs is predominantly caused by their paracrine actions rather than their EC transdifferentiation potential (Figure 25.3). In 2003, Al-Khaldi *et al.* demonstrated with LacZ transgenic BM-MSCs in the mouse Matrigel plug assay that over 99% of the generated blood vessels originated from host-derived ECs and that transdifferentiation of BM-MSCs toward ECs in that setting was very rare. In addition, only 10% of injected BM-MSCs were found in close proximity to, or in, blood vessels [70]. Another study using $GFP^+$ BM-MSCs in the treatment of wounds in diabetic $GFP^-$ mice showed that the rate of MSC engraftment 28 days after injection was less than 1%, and again no participation of the transplanted $GFP^+$ BM-MSCs into the recipients' vasculature was observed [26]. Although the low engraftment of MSCs in most settings and the lack of incorporation into recipient blood vessels is widely described, it cannot excluded that in particular settings or time frames the EC differentiation of MSCs may exist. A study by Yue *et al.* in which rat BM-MSCs were injected in a rat vein grafting model showed that the injected 4′,6-diamidino-2-phenylindole-labeled BM-MSCs differentiated into ECs 2 weeks after transplantation were capable of adhering to the injured vascular wall and showed immunopositivity for CD31 and endothelial nitric oxide synthase [147].

Despite the fact that differentiation of MSCs toward ECs especially *in vivo* remains a controversial subject, numerous attempts have been made to skew MSCs towards EC transdifferentiation (summarized in Table 25.2). MSC commitment to EC is usually demonstrated by the upregulation of typical EC surface molecules such as CD31, CD34, VEGFR1, VEGFR2, and vWF. Functional tests performed include *in vitro* tube formation in Matrigel and uptake of acetylated low-density lipoproteins (Ac-LDLs), although macrophages are also able to internalize these molecules [168]. Based only on these commonly used *in vitro* assays, it may be inaccurate to designate such differentiated MSCs as fully mature and functional ECs. It is more correct to describe these cells as EC-like cells. Although the *in vitro* differentiation approaches listed in Table 25.2 demonstrate indications for EC-like characteristics, such as EC marker expression, Matrigel tube formation, and Ac-LDL uptake, there is very little evidence that shows morphological and ultrastructural evidence of EC transdifferentiation. For example, the presence of typical EC characteristics such as Weibel–Palade bodies and tight junctions, which can be demonstrated by transmission electron microscopy, have never been reported for EC-directed MSCs except in the study by Jazayeri *et al.* [153]. Additionally, there is also no evidence that links the use of these EC-like MSCs in an *in vivo* setting with functional nonleaking blood vessels.

Attempts to differentiate MSCs to ECs using VEGF have been widely described (see Table 25.2) Several reports claimed EC differentiation of MSCs with increased expression of EC markers such as VEGFR1, VEGFR2, VE-cadherin, vascular cell adhesion molecule 1, and vWF and increased formation of capillary-like tubes in an *in vitro* Matrigel assay [69,153,155,156,166]. In contrast, Roobrouck *et al.* demonstrated that incubation of human BM-MSCs in basal medium containing VEGF significantly increased mRNA expression of CD34, VEGFR1, and VEGFR2, but not of TIE-2 or vWF, and the mRNA level of CD31 was even decreased. These BM-MSCs did not form a tubular network when plated onto Matrigel *in vitro*, thereby failing the functional test of full EC differentiation [72].

In addition to molecular mediators such as VEGF and Notch, physical cues have also been investigated for their ability to transdifferentiate MSCs to ECs. Such tools include mechanical stimuli [169], shear stress [149,160], and seeding the cells on elastic nanofiber hydrogels [170] or on to three-dimensional matrices [171,172]; and see Chapter 8. For example, placenta-derived MSCs subjected to shear stress for 24 h showed a significant increase in vWF and PECAM-1 expression, LDL uptake, and Matrigel tube formation [173].

Although the evidence for full differentiation of MSCs to ECs *in vitro* and *in vivo* is debatable, preconditioning MSCs in the so-called EC differentiation medium may still be valuable as this pretreatment might transform the MSCs toward a more angiogenic cell type. For example, placental MSCs preincubated with 50 ng/mL of VEGF displayed higher angiogenic activity than normal placental MSCs in the CAM assay [69]. AD-MSCs treated with an EC growth medium EGM-2 secreted higher amounts of Ang-1, promoted HUVEC migration, and significantly suppressed neointimal formation in a wire injury model of the rat femoral artery compared with control AD-MSCs [148]. Neointimal hyperplasia refers to the proliferation and migration of vascular smooth muscle cells primarily in the tunica intima, resulting in the thickening of arterial walls and decreased arterial lumen space. Neointimal hyperplasia is the major cause of restenosis after percutaneous coronary interventions such as stenting or angioplasty. The term neointima is used because the cells in the hyperplastic regions of the vascular wall have histological characteristics of both intima and normal artery cells.

## 25.7 Conclusions, therapeutic expectations, and challenges

During the last few years intensive research has led to the identification of a multitude of bioactive angiogenic molecules, such as VEGF, FGF-2, and MCP-1 in the CM of MSCs. In addition, the role of MSCs in several key events of angiogenesis, such as EC proliferation, tube formation, and stabilization, has been unraveled. MSCs from an ever-growing list of tissue sources have been shown to induce angiogenesis in various *in vitro* and

**Table 25.2** Different protocols of *in vitro* MSC differentiation toward cells with EC-like features.

| MSC type | Reference | Incubation time (days) | Differentiation medium |
|---|---|---|---|
| AD-MSC | [148] | 7 | EGM-2 + 5% FCS |
| | [149] | 18 | ECGS (14 days) + shear stress (4 days) |
| | [77] | 2 | On Matrigel, EGM-2 + 50 ng/mL VEGF + 10 ng/mL FGF-2 |
| | [150] | 12 | EGM-2 BulletKit (without the FGF-2) + ITS + 10 ng/mL FGF-2 |
| AM-MSC | [151] | 7 | 2%FCS and 50 ng/mL VEGF |
| BM-MSC | [152] | 12 | 5% FCS + 100 ng/mL VEGF + 50 ng/mL EGF + 1 μg/mL hydrocortisone |
| | [153] | 5 | 5% FCS, 50 ng/mL VEGF, and 20 ng/mL IGF-1 |
| | [154] | 21 | EGF + VEGF + hydrocortisone |
| | [155] | 21 | EGM-2 + 2% FCS |
| | [156] | 7 | 2% FCS + 50 ng/mL VEGF |
| | [157] | 11 | EGM-2 + shear stress (10 days) + 1 day on Matrigel |
| | [72] | 14 | Basal medium + 100 ng/mL VEGF |
| | [158] | 14 or 28 | Basal medium |
| | [159] | 21 | 5% FCS + 50 ng/mL VEGF |
| | [70] | 14 | 10% FCS, 50 ng/mL VEGF |
| | [160] | 7 | VEGF alone |
| | [160] | 8 | Shear stress (24 h) + VEGF (7 days) |
| | [161] | 5 | Co-culture with rabbit EC |
| | [162] | 7 | 2% FCS + 0.5 μM simvastatin |
| | [163] | 10 | EGM-2 + 50 ng/mL VEGF |
| DPSC | [78] | 10 | On Matrigel, EGM-2 |
| | [78] | 3 | EBM-2 + 2% serum + 10 ng/mL VEGF + 10 ng/mL FGF-2 |
| | [164] | 10 | 10% FCS + 1% ITS + 20 ng/mL VEGF |
| | [165] | 7 | 2% FCS and 50 ng/mL VEGF |
| DPSC-SHED | [166] | 28 | EGM-2 + 50 ng/mL VEGF |
| Placental MSC | [69] | 21 | EGM-2 + 2% FCS + 50 ng/mL VEGF |
| UM-MSC | [167] | 21 | EGF + VEGF + hydrocortisone |
| WJ-MSC | [152] | 12 | 5% FCS + 100 ng/mL VEGF + 50 ng/mL EGF + 1 μg/mL hydrocortisone |

*in vivo* models, and their promising preclinical potential has already been translated into successful phase I and II trials.

Some major hurdles remain, especially with regard to the heterogeneity of MSCs and the variability of their isolation and expansion protocols. The discovery of a unique and unambiguous MSC marker would be useful. Advances in cell labeling and imaging techniques should help resolve questions concerning homing and engrafting of administered MSCs. Large-scale production of MSCs in serum-free conditions in closed-system bioreactors is an important goal.

For angiogenesis-related diseases the paracrine actions of MSCs account for most of their observed therapeutic effect. The CM of MSCs may theoretically be able to substitute for the cells themselves, but it is likely that the interactions between MSCs and the injured target tissue are extremely complex with multiple cell-to-cell interactions and a number of mechanisms by which therapeutic benefit occurs taking place, making it unlikely that a soup of proteins will be an adequate substitute.

The occurrence and role of MSC-derived miRNAs involved in angiogenesis is currently relatively unexplored territory and warrants further examination.

In conclusion, in recent years significant advances have been made in unravelling the angiogenic capacities of MSCs. This is illustrated by successful application of MSCs and neovascularization in a wide variety of *in vivo* preclinical models of AMI, stroke, critical limb ischemia, and wound healing, resulting in an increasing optimism of both basic scientists and clinicians [29,32,37,151,166].

## References


1 Carmeliet P and Jain RK. Principles and mechanisms of vessel normalization for cancer and other angiogenic diseases. *Nat Rev Drug Discov* 2011;**10**:417–427.
2 Sieveking DP and Ng MK. Cell therapies for therapeutic angiogenesis: back to the bench. *Vasc Med* 2009;**14**:153–166.
3 Herbert SP and Stainier DY. Molecular control of endothelial cell behaviour during blood vessel morphogenesis. *Nat Rev Mol Cell Biol* 2011;**12**:551–564.
4 Potente M, Gerhardt H, and Carmeliet P. Basic and therapeutic aspects of angiogenesis. *Cell* 2011;**146**:873–887.
5 Ferrara N and Kerbel RS. *Angiogenesis as a therapeutic target*. *Nature* 2005;**438**:967–974.
6 Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* 1971;**285**:1182–1186.
7 Folkman J. Is angiogenesis an organizing principle in biology and medicine? *J Pediatr Surg* 2007;**42**:1–11.
8 Jain RK. Molecular regulation of vessel maturation. *Nat Med* 2003;**9**:685–693.
9 Carmeliet P and Jain RK. Molecular mechanisms and clinical applications of angiogenesis. *Nature* 2011;**473**:298–307.
10 De Spiegelaere W, Casteleyn C, Van den Broeck W, *et al*. Intussusceptive angiogenesis: a biologically relevant form of angiogenesis. *J Vasc Res* 2012;**49**:390–404.
11 Fang S and Salven P. Stem cells in tumor angiogenesis. *J Mol Cell Cardiol* 2011;**50**:290–295.
12 Gerhardt H, Golding M, Fruttiger M, *et al.* VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 2003;**161**:1163–1177.
13 Siekmann AF, Affolter M, and Belting HG. The tip cell concept 10 years after: new players tune in for a common theme. *Exp Cell Res* 2013;**319**:1255–1263.
14 Ribatti D and Crivellato E. "Sprouting angiogenesis", a reappraisal. *Dev Biol* 2012;**372**:157–165.
15 Welti J, Loges S, Dimmeler S, and Carmeliet P. Recent molecular discoveries in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest* 2013;**123**:3190–3200.
16 Schoors S, De Bock K, Cantelmo AR, *et al*. Partial and transient reduction of glycolysis by PFKFB3 blockade reduces pathological angiogenesis. *Cell Metab* 2014;**19**:37–48.
17 Ghesquière B, Wong BW, Kuchnio A, and Carmeliet P. Metabolism of stromal and immune cells in health and disease. *Nature* 2014;**511**:167–176.
18 De Bock K, Georgiadou M, Schoors S, *et al*. Role of PFKFB3-driven glycolysis in vessel sprouting. *Cell* 2013;**154**:651–663.
19 Bhadada SV, Goyal BR, and Patel MM. Angiogenic targets for potential disorders. *Fundam Clin Pharmacol* 2011;**25**:29–47.
20 Dragneva G, Korpisalo P, and Yla-Herttuala S. Promoting blood vessel growth in ischemic diseases: challenges in translating preclinical potential into clinical success. *Dis Model Mech* 2013;**6**:312–322.
21 Annex BH and Simons M. Growth factor-induced therapeutic angiogenesis in the heart: protein therapy. *Cardiovasc Res* 2005;**65**:649–655.
22 Yla-Herttuala S and Alitalo K. Gene transfer as a tool to induce therapeutic vascular growth. *Nat Med* 2003;**9**:694–701.
23 Laschke MW, Harder Y, Amon M, *et al.* Angiogenesis in tissue engineering: breathing life into constructed tissue substitutes. *Tissue Eng* 2006;**12**:2093–2104.
24 Caplan AI and Correa D. The MSC: an injury drugstore. *Cell Stem Cell* 2011;**9**:11–15.
25 Hung SC, Pochampally RR, Chen SC, *et al*. Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K–Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 2007;**25**:2363–2370.
26 Wu Y, Chen L, Scott PG, and Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007;**25**:2648–2659.
27 Estrada R, Li N, Sarojini H, *et al.* Secretome from mesenchymal stem cells induces angiogenesis via Cyr61. *J Cell Physiol* 2009;**219**:563–571.
28 Kinnaird T, Stabile E, Burnett MS, *et al.* Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 2004;**109**:1543–1549.
29 Tögel F, Weiss K, Yang Y, *et al.* Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol* 2007;**292**: F1626–F1635.
30 Rehman J, Traktuev D, Li J, *et al.* Secretion of angiogenic and antiapoptotic factors by human adipose stromal cells. *Circulation* 2004;**109**:1292–1298.
31 Liu SH, Huang JP, Lee RK, *et al.* Paracrine factors from human placental multipotent mesenchymal stromal cells protect endothelium from oxidative injury via STAT3 and manganese superoxide dismutase activation. *Biol Reprod* 2010;**82**:905–913.
32 Majumdar MK, Thiede MA, Mosca JD, *et al.* Phenotypic and functional comparison of cultures of marrow-derived mesenchymal stem cells (MSCs) and stromal cells. *J Cell Physiol* 1998;**176**: 57–66.
33 De Luca A, Gallo M, Aldinucci D, *et al.* Role of the EGFR ligand/receptor system in the secretion of angiogenic factors in mesenchymal stem cells. *J Cell Physiol* 2011;**226**:2131–2138.
34 Boomsma RA and Geenen DL. Mesenchymal stem cells secrete multiple cytokines that promote angiogenesis and have contrasting effects on chemotaxis and apoptosis. *PLoS ONE* 2012;**7**:e35685.
35 Son MR, Marquez-Curtis LA, Wysoczynski KM, *et al.* Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1-CXCR4 and hepatocyte growth factor-c-met axes and involves matrix metalloproteinases. *Stem Cells* 2006;**24**:1254–1264.
36 Bronckaers A, Hilkens P, Fanton Y, *et al.* Angiogenic properties of human dental pulp stem cells. *PLoS ONE* 2013;**8** (8):e71104.
37 Tran-Hung L, Laurent P, Camps J, and About I. Quantification of angiogenic growth factors released by human dental cells after injury. *Arch Oral Biol* 2008;**53**:9–13.
38 Han SK, Chun KW, Gye MS, and Kim WK. The effect of human bone marrow stromal cells and dermal fibroblasts on angiogenesis. *Plast Reconstr Surg* 2006;**117**:829–835.
39 Jadlowiec J, Dongell D, Smith J, *et al.* Pregnancy-associated plasma protein-A is involved in matrix mineralization of human adult mesenchymal stem cells and angiogenesis in the chick chorioallontoic membrane. *Endocrinology* 2005;**146**:3765–3772.

40 Kwon HM, Hur SM, Park KY, *et al.* Multiple paracrine factors secreted by mesenchymal stem cells contribute to angiogenesis. *Vascul Pharmacol* 2014;**63** (1):19–28.

41 Annabi B, Lee YT, Turcotte S, *et al.* Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. *Stem Cells* 2003;**21**:337–347.

42 Aranha AM, Zhang Z, Neiva KG, *et al.* Hypoxia enhances the angiogenic potential of human dental pulp cells. *J Endod* 2010;**36**:1633–1637.

43 Efimenko A, Starostina E, Kalinina N, and Stolzing A. Angiogenic properties of aged adipose derived mesenchymal stem cells after hypoxic conditioning. *J Transl Med* 2011;**9**:10.

44 Wen Z, Zheng S, Zhou C, *et al.* Bone marrow mesenchymal stem cells for post-myocardial infarction cardiac repair: microRNAs as novel regulators. *J Cell Mol Med* 2012;**16**:657–671.

45 Fasanaro P, D'Alessandra Y, Di Stefano V, *et al.* MicroRNA-210 modulates endothelial cell response to hypoxia and inhibits the receptor tyrosine kinase ligand Ephrin-A3. *J Biol Chem* 2008;**283**: 15878–15883.

46 Nicoli S, Standley C, Walker P, *et al.* MicroRNA-mediated integration of haemodynamics and Vegf signalling during angiogenesis. *Nature* 2010;**464**:1196–1200.

47 Wen Z, Huang W, Feng Y, *et al.* MicroRNA-377 regulates mesenchymal stem cell-induced angiogenesis in ischemic hearts by targeting VEGF. *PLoS ONE* 2014;**9**:e104666.

48 Lin RZ, Moreno-Luna R, Zhou B, *et al.* Equal modulation of endothelial cell function by four distinct tissue-specific mesenchymal stem cells. *Angiogenesis* 2012;**15**:443–455.

49 Choi M, Lee HS, Naidansaren P, *et al.* Proangiogenic features of Wharton's jelly-derived mesenchymal stromal/stem cells and their ability to form functional vessels. *Int J Biochem Cell Biol* 2013;**45**:560–570.

50 Gruber R, Kandler B, Holzmann P, *et al.* Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. *Tissue Eng* 2005;**11**:896–903.

51 Burlacu A, Grigorescu G, Rosca AM, *et al.* Factors secreted by mesenchymal stem cells and endothelial progenitor cells have complementary effects on angiogenesis *in vitro*. *Stem Cells Dev* 2013;**22**:643–653.

52 Hilkens P, Fanton Y, Martens W, *et al.* Pro-angiogenic impact of dental stem cells *in vitro* and *in vivo*. *Stem Cell Res* 2014;**12**:778–790.

53 Pedersen TO, Blois AL, Xue Y, *et al.* Mesenchymal stem cells induce endothelial cell quiescence and promote capillary formation. *Stem Cell Res Ther* 2014;**5**:23.

54 Menge T, Gerber M, Wataha K, *et al.* Human mesenchymal stem cells inhibit endothelial proliferation and angiogenesis via cell-cell contact through modulation of the VE-cadherin/β-catenin signaling pathway. *Stem Cells Dev* 2013;**22**:148–157.

55 Lee JK, Park SR, Jung BK, *et al.* Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* 2013;**8**:e84256.

56 Potapova IA, Gaudette GR, Brink PR, *et al.* Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells *in vitro*. *Stem Cells* 2007;**25**:1761–1768.

57 Ghajar CM, Chen X, Harris JW, *et al.* The effect of matrix density on the regulation of 3-D capillary morphogenesis. *Biophys J* 2008;**94** (5):1930–1941.

58 Kachgal S and Putnam AJ. Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms. *Angiogenesis* 2011;**14**:47–59.

59 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.

60 Duffy GP, Ahsan T, O'Brien T, *et al.* Bone marrow-derived mesenchymal stem cells promote angiogenic processes in a time- and dose-dependent manner *in vitro*. *Tissue Eng Part A* 2009;**15**:2459–2470.

61 Sorrell JM, Baber MA, and Caplan AI. Influence of adult mesenchymal stem cells on *in vitro* vascular formation. *Tissue Eng Part A* 2009;**15**:1751–1761.

62 Au P, Tam J, Fukumura D, and Jain RK. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* 2008;**111**:4551–4558.

63 McFerrin HE, Olson SD, Gutschow MV, *et al.* Rapidly self-renewing human multipotent marrow stromal cells (hMSC) express sialyl Lewis X and actively adhere to arterial endothelium in a chick embryo model system. *PLoS ONE* 2014;**9**:e105411.

64 Ball SG, Bayley C, Shuttleworth CA, and Kielty CM. Neuropilin-1 regulates platelet-derived growth factor receptor signalling in mesenchymal stem cells. *Biochem J* 2010;**427**:29–40.

65 Otsu K, Das S, Houser SD, *et al.* Concentration-dependent inhibition of angiogenesis by mesenchymal stem cells. *Blood* 2009;**113**: 4197–4205.

66 Ribatti D. Chick embryo chorioallantoic membrane as a useful tool to study angiogenesis. *Int Rev Cell Mol Biol* 2008;**270**:181–224.

67 Liu Y, Han ZP, Zhang SS, *et al.* Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem* 2011;**286**:25007–25015.

68 Oskowitz A, McFerrin H, Gutschow M, *et al.* Serum-deprived human multipotent mesenchymal stromal cells (MSCs) are highly angiogenic. *Stem Cell Res* 2011;**6**:215–225.

69 Lee MY, Huang JP, Chen YY, *et al.* Angiogenesis in differentiated placental multipotent mesenchymal stromal cells is dependent on integrin $\alpha_5\beta_1$. *PLoS ONE* 2009;**4**:e6913.

70 Auerbach R, Lewis R, Shinners B, *et al.* Angiogenesis assays: a critical overview. *Clin Chem* 2003;**49**:32–40.

71 Al-Khaldi A, Eliopoulos N, Martineau D, *et al.* Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response *in vivo*. *Gene Ther* 2003;**10**:621–629.

72 Roobrouck VD, Clavel C, Jacobs SA, *et al.* Differentiation potential of human postnatal mesenchymal stem cells, mesoangioblasts, and multipotent adult progenitor cells reflected in their transcriptome and partially influenced by the culture conditions. *Stem Cells* 2011;**29**:871–882.

73 Janebodin K, Zeng Y, Buranaphatthana W, *et al.* VEGFR2-dependent angiogenic capacity of pericyte-like dental pulp stem cells. *J Dent Res* 2013;**92**:524–531.

74 Ouriel K. Peripheral arterial disease. *Lancet* 2001;**358**:1257–1264.

75 Iwase T, Nagaya N, Fujii T, *et al.* Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc Res* 2005;**66**:543–551.

76 Al-Khaldi A, Al-Sabti H, Galipeau J, and Lachapelle K. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. *Ann Thorac Surg* 2003;**75**:204–209.

77 Moon MH, Kim SY, Kim YJ, *et al.* Human adipose tissue-derived mesenchymal stem cells improve postnatal neovascularization in a mouse model of hindlimb ischemia. *Cell Physiol Biochem* 2006;**17**:279–290.

78 Iohara K, Zheng L, Wake H, *et al.* A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp. *Stem Cells* 2008;**26**:2408–2418.

79 Schiavetta A, Maione C, Botti C, *et al.* A phase II trial of autologous transplantation of bone marrow stem cells for critical limb ischemia: results of the Naples and Pietra Ligure Evaluation of Stem Cells study. *Stem Cells Transl Med* 2012;**1**:572–578.

80 Kalladka D and Muir KW. Brain repair: cell therapy in stroke. *Stem Cells Cloning* 2014;**7**:31–44.

81 Lozano R, Naghavi M, Foreman K, *et al.* Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. *Lancet* 2012;**380**:2095–2128.

82 Gervois P, Struys T, Hilkens P, *et al.* Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons. *Stem Cells Dev* 2015;**24** (3):296–311.

83 Barnabé GF, Schwindt TT, Calcagnotto ME, *et al.* Chemically-induced RAT mesenchymal stem cells adopt molecular properties of neuronal-like cells but do not have basic neuronal functional properties. *PLoS ONE* 2009;**4**:e5222.

84 De Vocht N, Praet J, Reekmans K, *et al.* Tackling the physiological barriers for successful mesenchymal stem cell transplantation into the central nervous system. *Stem Cell Res Ther* 2013;**4** (4):101.

85 Mora-Lee S, Sirerol-Piquer MS, Gutiérrez-Pérez M, *et al.* Therapeutic effects of hMAPC and hMSC transplantation after stroke in mice. *PLoS ONE* 2012;**7**:e43683.

86 Banerjee S, Williamson DA, Habib N, and Chataway J. The potential benefit of stem cell therapy after stroke: an update. *Vasc Health Risk Manag* 2012;**8**:569–580.

87 Eckert MA, Vu Q, Xie K, *et al.* Evidence for high translational potential of mesenchymal stromal cell therapy to improve recovery from ischemic stroke. *J Cereb Blood Flow Metab* 2013;**33**:1322–1334.

88 Chen J, Zhang ZG, Li Y, *et al.* Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ Res* 2003;**92**:692–699.

89 Ma XL, Liu KD, Li FC, *et al.* Human mesenchymal stem cells increases expression of alpha-tubulin and angiopoietin 1 and 2 in focal cerebral ischemia and reperfusion. *Curr Neurovasc Res* 2013;**10**:103–111.

90 Jeon D, Chu K, Lee ST, *et al.* Neuroprotective effect of a cell-free extract derived from human adipose stem cells in experimental stroke models. *Neurobiol Dis* 2013;**54**:414–420.

91 Bang OY, Lee JS, Lee PH, and Lee G. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005;**57**:874–882.

92 Lee JS, Hong JM, Moon GJ, *et al.* A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 2010;**28**:1099–1106.

93 Honmou O, Houkin K, Matsunaga T, *et al.* Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 2011;**134**:1790–1807.

94 Russo V, Young S, Hamilton A, *et al.* Mesenchymal stem cell delivery strategies to promote cardiac regeneration following ischemic injury. *Biomaterials* 2014;**35**:3956–3974.

95 Van der Spoel TI, Jansen of Lorkeers SJ, Agostoni P, *et al.* Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. *Cardiovasc Res* 2011;**91**:649–658.

96 Gandia C, Armiñan A, García-Verdugo JM, *et al.* Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction. *Stem Cells* 2008;**26**:638–645.

97 Dai W, Hale SL, Martin BJ, *et al.* Allogeneic mesenchymal stem cell transplantation in postinfarcted rat myocardium: short- and long-term effects. *Circulation* 2005;**112**:214–223.

98 Uemura R, Xu M, Ahmad N, and Ashraf M. Bone marrow stem cells prevent left ventricular remodeling of ischemic heart through paracrine signaling. *Circ Res* 2006;**98**:1414–1421.

99 Ranganath SH, Levy O, Inamdar MS, and Karp JM. *Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease*. *Cell Stem Cell* 2012;**10**:244–258.

100 Miyahara Y, Nagaya N, Kataoka M, *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* 2006;**12**:459–465.

101 Kim SW, Zhang HZ, Kim CE, *et al.* Amniotic mesenchymal stem cells with robust chemotactic properties are effective in the treatment of a myocardial infarction model. *Int J Cardiol* 2013;**168** (2):1062–1069.

102 Markel TA, Wang Y, Herrmann JL, *et al.* VEGF is critical for stem cell-mediated cardioprotection and a crucial paracrine factor for defining the age threshold in adult and neonatal stem cell function. *Am J Physiol Heart Circ Physiol* 2008;**295**:H2308–H2314.

103 Shabbir A, Zisa D, Lin H, *et al.* Activation of host tissue trophic factors through JAK-STAT3 signaling: a mechanism of mesenchymal stem cell-mediated cardiac repair. *Am J Physiol Heart Circ Physiol* 2010;**299**:H1428–H1438.

104 Tongers J, Losordo DW, and Landmesser U. Stem and progenitor cell-based therapy in ischaemic heart disease: promise, uncertainties, and challenges. *Eur Heart J* 2011;**32**:1197–1206.

105 Assmus B, Schachinger V, Teupe C, *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* 2002;**106**:3009–3017.

106 Meyer GP, Wollert KC, Lotz J, *et al.* Intracoronary bone marrow cell transfer after myocardial infarction: eighteen months' follow-up data from the randomized, controlled BOOST (BOne marrOw transfer to enhance ST-elevation infarct regeneration) trial. *Circulation* 2006;**113**:1287–1294.

107 Schächinger V, Erbs S, Elsässer A, *et al.* Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J* 2006;**27**:2775–2783.

108 Lunde K, Solheim S, Aakhus S, *et al.* Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. *N Engl J Med* 2006;**355**:1199–1209.

109 Roncalli J, Mouquet F, Piot C, *et al.* Intracoronary autologous mononucleated bone marrow cell infusion for acute myocardial infarction: results of the randomized multicenter BONAMI trial. *Eur Heart J* 2011;**32**:1748–1757.

110 Janssens S, Dubois C, Bogaert J, *et al.* Autologous bone marrow-derived stem-cell transfer in patients with ST-segment elevation myocardial infarction: double-blind, randomised controlled trial. *Lancet* 2006;**367**:113–121.

111 Leistner DM, Fischer-Rasokat U, Honold J, *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI): final 5-year results suggest long-term safety and efficacy. *Clin Res Cardiol* 2011;**100**:925–934.

112 Meyer GP, Wollert KC, Lotz J, *et al.* Intracoronary bone marrow cell transfer after myocardial infarction: 5-year follow-up from the randomized-controlled BOOST trial. *Eur Heart J* 2009;**30**: 2978–2984.

113 Perin EC, Sanz-Ruiz R, Sánchez PL, *et al.* Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: the PRECISE trial. *Am Heart J* 2014;**168**:88–95.e2.

114 Houtgraaf JH, den Dekker WK, van Dalen BM, *et al.* First experience in humans using adipose tissue-derived regenerative cells in the treatment of patients with ST-segment elevation myocardial infarction. *J Am Coll Cardiol* 2012;**59**:539–540.

115 Qayyum AA, Haack-Sørensen M, Mathiasen AB, *et al.* Adipose-derived mesenchymal stromal cells for chronic myocardial ischemia (MyStromalCell Trial): study design. *Regen Med* 2012;**7**:421–428.

116 Davey GC, Patil SB, O'Loughlin A, and O'Brien T. Mesenchymal stem cell-based treatment for microvascular and secondary complications of diabetes mellitus. *Front Endocrinol (Lausanne)* 2014;**5**:86.

117 Rajashekhar G. Mesenchymal stem cells: new players in retinopathy therapy. *Front Endocrinol (Lausanne)* 2014;**5**:59.

118 Hocking AM and Gibran NS. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp Cell Res* 2010;**316**:2213–2219.

119 Lin YC, Grahovac T, Oh SJ, *et al.* Evaluation of a multi-layer adipose-derived stem cell sheet in a full-thickness wound healing model. *Acta Biomater* 2013;**9**:5243–52450.

120 Chen L, Tredget EE, Wu PY, and Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3**:e1886.

121 Lee EY, Xia Y, Kim WS, *et al.* Hypoxia-enhanced wound-healing function of adipose-derived stem cells: increase in stem cell proliferation and up-regulation of VEGF and bFGF. *Wound Repair Regen* 2009;**17**:540–547.

122 Sasaki M, Abe R, Fujita Y, *et al.* Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol* 2008;**180**:2581–2587.

123 Falanga V, Iwamoto S, Chartier M, *et al.* Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. *Tissue Eng* 2007;**13**:1299–1312.

124 Dash NR, Dash SN, Routray P, *et al.* Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuvenation Res* 2009;**12**:359–366.

125 Kirana S, Stratmann B, Prante C, *et al.* Autologous stem cell therapy in the treatment of limb ischaemia induced chronic tissue ulcers of diabetic foot patients. *Int J Clin Pract* 2012;**66**:384–393.

126 Cuiffo BG and Karnoub AE. Mesenchymal stem cells in tumor development: emerging roles and concepts. *Cell Adh Migr* 2012;**6**:220–230.

127 Klopp AH, Gupta A, Spaeth E, *et al.* Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells* 2011;**29**:11–19.

128 Jeon ES, Lee IH, Heo SC, *et al.* Mesenchymal stem cells stimulate angiogenesis in a murine xenograft model of A549 human adenocarcinoma through an LPA1 receptor-dependent mechanism. *Biochim Biophys Acta* 2010;**1801**:1205–1213.

129 Djouad F, Plence P, Bony C, *et al.* Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;**102**:3837–3844.

130 Galiè M, Konstantinidou G, Peroni D, *et al.* Mesenchymal stem cells share molecular signature with mesenchymal tumor cells and favor early tumor growth in syngeneic mice. *Oncogene* 2008;**27**:2542–2551.

131 Suzuki K, Sun R, Origuchi M, *et al.* Mesenchymal stromal cells promote tumor growth through the enhancement of neovascularization. *Mol Med* 2011;**17**:579–587.

132 Huang WH, Chang MC, Tsai KS, *et al.* Mesenchymal stem cells promote growth and angiogenesis of tumors in mice. *Oncogene* 2013;**32**:4343–4354.

133 Zhu W, Huang L, Li Y, *et al.* Mesenchymal stem cell-secreted soluble signaling molecules potentiate tumor growth. *Cell Cycle* 2011;**10**:3198–3207.

134 Khakoo AY, Pati S, Anderson SA, *et al.* Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med* 2006;**203**:1235–1247.

135 Qiao L, Xu Z, Zhao T, *et al.* Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Res* 2008;**18**:500–507.

136 Cousin B, Ravet E, Poglio S, *et al.* Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both *in vitro* and *in vivo*. *PLoS ONE* 2009;**4**:e6278.

137 Dasari VR, Kaur K, Velpula KK, *et al.* Downregulation of focal adhesion kinase (FAK) by cord blood stem cells inhibits angiogenesis in glioblastoma. *Aging (Albany NY)* 2010;**2**:791–803.

138 Komarova S, Kawakami Y, Stoff-Khalili MA, *et al.* Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* 2006;**5**:755–766.

139 Niess H, Bao Q, Conrad C, *et al.* Selective targeting of genetically engineered mesenchymal stem cells to tumor stroma microenvironments using tissue-specific suicide gene expression suppresses growth of hepatocellular carcinoma. *Ann Surg* 2011;**254**:767–774, discussion 774–775.

140 Karnoub AE, Dash AB, Vo AP, *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**:557–563.

141 Dwyer RM, Khan S, Barry FP, *et al.* Advances in mesenchymal stem cell-mediated gene therapy for cancer. *Stem Cell Res Ther* 2010;**1**:25.

142 Nakamizo A, Marini F, Amano T, *et al.* Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;**65**:3307–3318.

143 Van Eekelen M, Sasportas LS, Kasmieh R, *et al.* Human stem cells expressing novel TSP-1 variant have anti-angiogenic effect on brain tumors. *Oncogene* 2010;**29**:3185–3195.

144 Lalu MM, McIntyre L, Pugliese C, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**:e47559.

145 Liu GS, Peshavariya HM, Higuchi M, *et al.* Pharmacological priming of adipose-derived stem cells for paracrine VEGF

production with deferoxamine. *J Tissue Eng Regen Med* 2016;**10** (3): E167–E176.

146 Crisostomo PR, Wang Y, Markel TA, *et al.* Human mesenchymal stem cells stimulated by TNF-α, LPS, or hypoxia produce growth factors by an NFκB- but not JNK-dependent mechanism. *Am J Physiol Cell Physiol* 2008;**294**:C675–C682.

147 Yue WM, Liu W, Bi YW, *et al.* Mesenchymal stem cells differentiate into an endothelial phenotype, reduce neointimal formation, and enhance endothelial function in a rat vein grafting model. *Stem Cells Dev* 2008;**17**:785–93.

148 Takahashi M, Suzuki E, Oba S, *et al.* Adipose tissue-derived stem cells inhibit neointimal formation in a paracrine fashion in rat femoral artery. *Am J Physiol Heart Circ Physiol* 2010;**298**: H415–H423.

149 Fischer LJ, McIlhenny S, Tulenko T, *et al.* Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. *J Surg Res* 2009;**152**:157–166.

150 Konno M, Hamazaki TS, Fukuda S, *et al.* Efficiently differentiating vascular endothelial cells from adipose tissue-derived mesenchymal stem cells in serum-free culture. *Biochem Biophys Res Commun* 2010;**400**:461–465.

151 Alviano F, Fossati V, Marchionni C, *et al.* Term amniotic membrane is a high throughput source for multipotent mesenchymal stem cells with the ability to differentiate into endothelial cells *in vitro*. *BMC Dev Biol* 2007;**7**:11.

152 Chen MY, Lie PC, Li ZL, Wei X. Endothelial differentiation of Wharton's jelly-derived mesenchymal stem cells in comparison with bone marrow-derived mesenchymal stem cells. *Exp Hematol* 2009;**37**:629–640.

153 Jazayeri M, Allameh A, Soleimani M, *et al.* Molecular and ultrastructural characterization of endothelial cells differentiated from human bone marrow mesenchymal stem cells. *Cell Biol Int* 2008;**32**:1183–1192.

154 Chung N, Jee BK, Chae SW, *et al.* HOX gene analysis of endothelial cell differentiation in human bone marrow-derived mesenchymal stem cells. *Mol Biol Rep* 2009;**36**:227–235.

155 Liu JW, Dunoyer-Geindre S, Serre-Beinier V, *et al.* Characterization of endothelial-like cells derived from human mesenchymal stem cells. *J Thromb Haemost* 2007;**5**:826–834.

156 Oswald J, Boxberger S, Jørgensen B, *et al.* Mesenchymal stem cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 2004;**22**:377–384.

157 Portalska KJ, Chamberlain MD, Lo C, *et al.* Collagen modules for in situ delivery of mesenchymal stromal cell-derived endothelial cells for improved angiogenesis. J Tissue Eng Regen Med 2013; doi: 10.1002/term.1738.

158 Whyte JL, Ball SG, Shuttleworth CA, *et al.* Density of human bone marrow stromal cells regulates commitment to vascular lineages. *Stem Cell Res* 2011;**6**:238–250.

159 Zhang G, Zhou J, Fan Q, *et al.* Arterial–venous endothelial cell fate is related to vascular endothelial growth factor and Notch status during human bone mesenchymal stem cell differentiation. *FEBS Lett* 2008;**582**:2957–2964.

160 Bai K, Huang Y, Jia X, *et al.* Endothelium oriented differentiation of bone marrow mesenchymal stem cells under chemical and mechanical stimulations. *J Biomech* 2010;**43**:1176–1181.

161 Wu X, Huang L, Zhou Q, *et al.* Mesenchymal stem cells participating in ex vivo endothelium repair and its effect on vascular smooth muscle cells growth. *Int J Cardiol* 2005;**105**:274–282.

162 Xu J, Liu X, Chen J, *et al.* Simvastatin enhances bone marrow stromal cell differentiation into endothelial cells via notch signaling pathway. *Am J Physiol Cell Physiol* 2009;**296**:C535–C543.

163 Pankajakshan D, Kansal V, and Agrawal DK. *In vitro* differentiation of bone marrow derived porcine mesenchymal stem cells to endothelial cells. *J Tissue Eng Regen Med* 2013;**7**:911–920.

164 Karbanová J, Soukup T, Suchánek J, *et al.* Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium. *Cells Tissues Organs* 2011;**193**:344–365.

165 Marchionni C, Bonsi L, Alviano F, *et al.* Angiogenic potential of human dental pulp stromal (stem) cells. *Int J Immunopathol Pharmacol* 2009;**22**:699–706.

166 Bento LW, Zhang Z, Imai A, *et al.* Endothelial differentiation of SHED requires MEK1/ERK signaling. *J Dent Res* 2013;**92**:51–57.

167 Gang EJ, Jeong JA, Han S, *et al. In vitro* endothelial potential of human UC blood-derived mesenchymal stem cells. *Cytotherapy* 2006;**8**:215–227.

168 Voyta JC, Via DP, Butterfield CE, and Zetter BR. Identification and isolation of endothelial cells based on their increased uptake of acetylated-low density lipoprotein. *J Cell Biol* 1984;**99**:2034–2040.

169 Maul TM, Chew DW, Nieponice A, and Vorp DA. Mechanical stimuli differentially control stem cell behavior: morphology, proliferation, and differentiation. *Biomech Model Mechanobiol* 2011;**10**:939–953.

170 Wingate K, Bonani W, Tan Y, *et al.* Compressive elasticity of three-dimensional nanofiber matrix directs mesenchymal stem cell differentiation to vascular cells with endothelial or smooth muscle cell markers. *Acta Biomater* 2012;**8**:1440–1449.

171 Zhang G, Drinnan CT, Geuss LR, and Suggs LJ. Vascular differentiation of bone marrow stem cells is directed by a tunable three-dimensional matrix. *Acta Biomater* 2010;**6**:3395–3403.

172 Valarmathi MT, Davis JM, Yost MJ, *et al.* A three-dimensional model of vasculogenesis. *Biomaterials* 2009;**30**:1098–1112.

173 Wu CC, Chao YC, Chen CN, *et al.* Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells. *J Biomech* 2008;**41**:813–821.

CHAPTER 26

# The relationship between mesenchymal stromal cells and endothelial cells

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## 26.1 Introduction

Mesenchymal stromal cells (MSCs) are considered important adult stem/stromal cells with a potential therapeutic role in a variety of diseases. In this context, bilateral interactions between MSCs and endothelial cells (ECs) are deemed to be of strategic relevance for the regulation of MSC function under physiologic and pathologic circumstances. Indeed, different and sometimes opposite roles are observed when MSCs are used for apparently similar purposes, in which MSC–EC interactions determine the kind of MSC behavior. For example, both stimulatory and inhibitory effects have been noted when MSCs were used for angiogenesis purposes. Understanding the molecular mechanisms that are involved in the relationship between MSCs and ECs will help to optimize therapeutic applications of these cells in regenerative medicine. In this chapter we attempt to shed further light on mechanisms of MSC–EC interactions in different physiological and pathological conditions and under specific spatiotemporal situations.

## 26.2 Transendothelial migration of mesenchymal stromal cells

### 26.2.1 Mesenchymal stromal cell adhesion to endothelial cells

An important issue is to how best to deliver MSCs to their target tissues. A number of different routes have been used, including direct administration into injured tissue and systemic administration in both preclinical animal studies and in clinical trials [1,2]. However, the precise molecular mechanisms by which MSCs migrate into their target tissues have not yet been fully elucidated.

A number of experimental studies have been conducted in an effort to illuminate the essential features of MSC trafficking and biodistribution into intact and injured tissues. It has been demonstrated that MSCs can adhere to the endothelium and extravasate at the site of inflammation by a leukocyte-like

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mechanism directed by an interaction between MSCs and ECs through selectin-mediated rolling, chemokine-derived retention, and integrin-dependent arrest on the endothelium [3,4]. There is a complex interplay between MSCs and ECs in different conditions [5]. It has been well established *in vivo* and *in vitro* that MSCs and ECs are able to influence the behavior of each other via ligand–receptor interactions [6].

A vast array of cell surface transmembrane molecules, such as integrins, is involved in MSC–EC adhesion during migration and homing of MSCs to the site of injury [7]. Integrins, as eukaryotic cell surface receptors, consist of heterodimers of α and β subunits, in which eight different α chains ($\alpha_1$ to $\alpha_8$) interact with at least 16 different β subunits ($\beta_1$ to $\beta_{16}$) to constitute approximately 22 different α–β heterodimers with different functions [8]. Each subunit contains a large extracellular and a small cytoplasmic domain [8]. The α–β heterodimer complexes with cell-membrane-spanning domains mediate dynamic cell–matrix and cell–cell linkages capable of transmitting inside-out and outside-in signals, which ultimately regulate numerous cellular responses and downstream signaling events such as cell growth, differentiation, and survival [9,10].

It has been shown that MSCs express several β ($\beta_1$ to $\beta_3$) and α ($\alpha_1$ to $\alpha_6$, and $\alpha_V$) subunits of integrins (see Figure 26.1A). On reaching confluency in cell culture, MSC expression of $\beta_3$, $\alpha_1$, $\alpha_3$, $\alpha_5$, and $\alpha_V$ integrins is increased, whereas the expression of $\alpha_6$ is decreased [7] (Figure 26.1A).

Specific integrins play a role in the adhesion of MSCs to a variety of human vascular ECs, including pulmonary arteries ($\beta_1$, $\alpha_V$, and $\alpha_X$), cardiac-derived microvasculature ($\beta_2$, $\alpha_X$, $\alpha_7$, and $\alpha_D$), and umbilical veins ($\beta_1$, $\beta_2$, and $\beta_3$) (Figure 26.1B). Neutralizing antibodies against the integrins of the β subclass can reduce MSC adherence to all the types of ECs mentioned here [7]. Moreover, selective blockade of integrin $\beta_1$ by specific neutralizing antibodies resulted in a significant reduction in the total number of MSCs in a preclinical model of myocardial infarction [11]. It has also been demonstrated that osteopontin-induced rat MSCs specifically required integrin $\beta_1$ for migration to different tissues in response to injury and inflammation [12]. The authors showed that osteopontin promoted integrin $\beta_1$ expression by MSCs in a concentration-dependent manner. Another study by Veevers-Lowe *et al.* showed that crosstalk between extracellular matrix (ECM)-associated receptors and distinct integrins on MSCs participated in the oriented migration of MSCs [13]. $\alpha_5\beta_1$ integrin adhesion to ECM components, especially fibronectin, triggered MSC migration via activating platelet-derived growth factor receptor (PDGFR)-β signaling with simultaneous focal adhesion kinase (FAK)-dependent phosphatidylinositide 3-kinase (PI3K) activation, actin reorganization, and membrane ruffling in MSCs, which occurred even in the absence of growth factor stimulation [13]. In this context, phosphorylated PDGFR-β accumulated in the leading edge of MSCs and temporarily co-localized with $\alpha_5\beta_1$-integrin at the apex of focal adhesion. The authors concluded that fibronectin-rich matrices might prepare PDGFR-β to recruit MSCs to sites of vascular remodeling [13]. Integrin-dependent intracellular signaling was also thought to be induced in leukemic cells in an MSC–leukemic cell co-culture system, which was mediated by integrin-linked kinase that phosphorylated Akt in a PI3K-dependent manner [14].

Among cell-adhesion-related molecules the Notch signaling axis has been shown to be critically important in direct cell–cell communication, which is essential for the regulated proliferation, apoptosis and fate decision of stem/progenitor cells during development [15]. Notch receptors Notch-1 to -4 are type I transmembrane proteins, with five structurally similar ligands: Delta-like-1, -3, -4 (DLL-1, DLL-3, DLL-4) and Jagged-1 and Jagged-2. Jagged-1 and Jagged-2 are other types of ligand/receptors that provoke MSC–EC interaction [16] (Figure 26.1B). The Notch-1, -2, and -3 receptors and the Jagged-1 ligand were shown to be expressed at significant levels in MSCs [16]. Notch-1 was found to increase the proliferation (documented by more Ki-$67^+$ cells and less cleaved Caspase$3^+$ apoptotic cells). In addition, it recruited MSCs to infarcted murine myocardium [17]. Xie *et al.* found that blocking Notch signaling by knockout of the transcription factor recombination signal binding protein-J (RBP-J) contributed to the upregulation of CXCR4 expression in MSCs and increased MSC migration in response to stromal cell-derived factor (SDF)-1α both *in vitro* and *in vivo* [18]. RBP-J is a DNA-binding protein that is a signal mediator from the Notch receptors after association with the Notch intracellular domain, or inhibition of y-secretase, an enzyme that releases RBP-J by a proteolytic mechanism upon triggering of the receptor. Notch signaling played a critical role in the recruitment of bone marrow MSCs to ischemic myocardium, which was essential for cardiac repair [19]. Bone marrow transplantation from mice with a hemizygous deletion of Notch-1 into normal mice resulted in a larger infarct size with reduction of recruitment, proliferation, and survival of MSCs [19].

The activation of MSCs by tumor necrosis factor (TNF)-α and interleukin (IL)-1 treatment before their injection significantly enhanced adhesion to the cardiac endothelium and promoted the homing of MSCs via intercellular cell adhesion molecule (ICAM)-1/lymphocyte function-associated antigen 1 (LFA-1) and G protein-coupled receptor signaling [4,20,21] (Figure 26.1B). Pretreatment of murine MSCs with interferon-γ (INF-γ) prior to intraperitoneal administration enhanced cell recruitment, possibly through upregulation of constitutively expressed chemokines and chemokine receptors, and alleviated the complications of experimentally induced colitis in mice [22,23]. It was demonstrated that MSCs underwent coordinated rolling on ECs and then adhered to ECs under shear flow by the mediation of selectins [24]. The authors reported that preincubation of human umbilical vein ECs (HUVECs) with a blocking antibody against P-selectin strongly decreased MSC binding to ECs, indicating that MSCs might bind to P-selectin using a fucose and sialic-acid-containing ligand [24]. As human MSCs do not express detectable levels of P-selectin glycoprotein ligand 1 (PSGL-1) and preincubation of MSCs with the antibody against

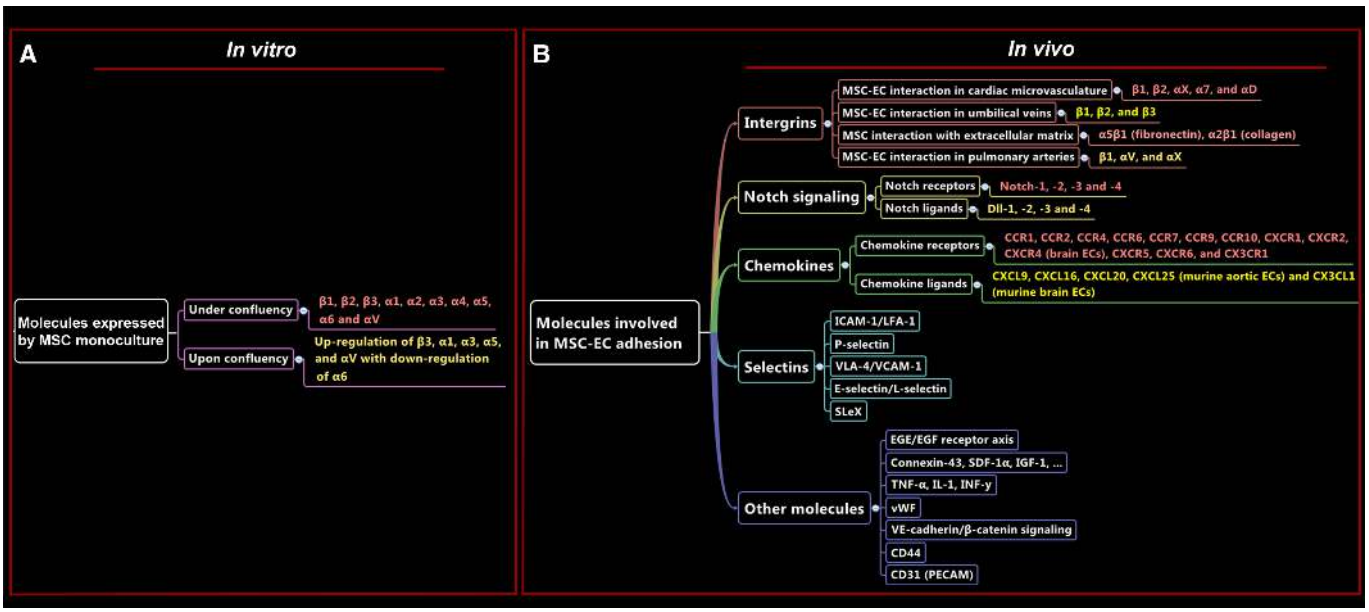



**Figure 26.1** (A) Molecules involved in MSC–EC interactions. A series of integrins are expressed by MSCs in *in vitro* culture conditions. (B) Within the body a large number of ligands and cognate receptors, including selectins, integrins, chemokines, and various signaling pathways, are involved in MSC–EC interactions in different physiological and pathological conditions. (See color plate section.)

PSGL-1 did not block the binding of MSCs to the surface of ECs, P-selectin-mediated MSC–EC binding appears to be independent of PSGL-1 [25]. Preincubation of ECs with anti-VCAM-1 (VCAM-1: vascular cell adhesion molecule 1) or of MSCs with anti-VLA-4 (VLA-4: very late activation antigen-4) prevented the adhesion of MSCs to ECs [24]. The authors concluded that MSCs bound to ECs in a P-selectin-dependent manner both *in vitro* and *in vivo*, and that CD44 along with VLA-4/VCAM-1 was engaged in the rolling of MSCs on ECs [25,26]. In support of these findings, coating the surface of MSCs with selectin-associated ligands such as sialyl Lewis X reduced the rolling velocity of these cells on an inflamed endothelial surface and enhanced the homing capacity of MSCs to the inflamed tissue [27].

Additionally, an increase in the level of expression of the cell surface molecule CD44, which is the receptor for hyaluronic acid, by platelet-derived growth factor (PDGF) stimulation of MSCs facilitated the migration of these cells through interaction with extracellular hyaluronic acid [28]. In addition, MSCs engineered with P- or L-selectin-binding nucleic acid aptamers efficiently adhered to the respective selectin surfaces of ECs and leukocytes [24]. (Aptamers are oligonucleotide or peptide molecules that bind to a specific target molecule.)

Sackstein *et al.* reported that a CD44 glycoform bearing α-2, 3-sialyl modifications was expressed on human MSCs but that these cells lacked E-selectin ligands and CXCR4, which limited tropism to bone tissue [29]. Modification of native CD44 to convert this molecule into an E-selectin/L-selectin ligand conferred potent binding capacity and tropism to bone via E-selectin-expressing marrow blood vessels [29].

Potapova *et al.* [30] demonstrated that treatment of ECs with von Willebrand factor (vWF) resulted in activation of intracellular downstream extracellular signal-regulated kinase (ERK)-1, −2, and p38 mitogen-activated protein kinase (MAPK) pathways with no effect on the gene or cell surface expression of E-selectin, P-selectin, VCAM-1, or ICAM-1. They showed that the activation of p38 MAPK in ECs by vWF could instigate MSC–EC adhesion [30]. VCAM-1/VLA-4 as well as $\beta_1$ integrins and matrix metalloproteinase (MMP)-2 play key roles in the trans-endothelial migration of MSCs [30] (Figure 26.1B). Moreover, lumican, a 37 kDa core protein proteoglycan belonging to the small leucine-rich proteoglycans family, was shown to inhibit MSC migration by a concomitant decrease of MMP-14 expression and activity, whereas the overexpression of MMP-14 improved the migratory potential of human MSCs *in vitro* [31]. Adding MSCs to EC tubes on Matrigel resulted in MSC–EC intercalation via development of connexin-43-based intercellular gap junctions [32]. Some MSC-derived secretory soluble factors, such as SDF-1α and insulin-like growth factor (IGF)-1, upregulated connexin-43 and facilitated the functional mobilization of MSCs into target tissues [33].

### 26.2.2 Trans-endothelial migration

A critical step for the successful recruitment and engraftment of systemically infused MSCs into damaged tissues is trans-endothelial migration. The exit of MSCs from the circulation requires their crossing the endothelial barrier posed by the vessel wall [4]. Moreover, the recruitment of tissue-specific MSCs (e.g., MSCs residing within the bone marrow) requires early entry into the circulation (intravasation) and subsequent transmigration across the endothelial barrier into inflamed or ischemic tissues (extravasation) [34]. Understanding bidirectional signaling cascades that regulate intravasation and extravasation during the trans-endothelial migration of MSCs will be helpful for optimization of the therapeutic use of MSCs.

The firm adhesion of MSCs to ECs is followed by endothelial transmigration via platelet-EC adhesion molecule-1 (PECAM-1/CD31), junctional adhesion molecules, and cadherins [4,35,36]. PECAM-1, a member of the immunoglobulin gene superfamily, is expressed on the surface of ECs, platelets, and hematopoietic cells and plays a pivotal role in leukocyte extravasation, modulation of integrin activity on leukocytes and T cells, and in angiogenesis [37]. Vascular endothelial cadherin (VE-cadherin)–β-catenin interaction with concurrent localization of PECAM-1 at the site of the cell–cell junction is essential for normal functioning of PECAM-1-derived signaling [38] (see Figure 26.1B). VE-cadherin is a key molecule responsible for the formation of strong intercellular junctions between ECs and connects to the underlying actin cytoskeleton through cytoplasmic interactions with the armadillo family proteins β-catenin, γ-catenin, and α-catenin [39].

As with leukocytes, MSCs transmigrate by a para-cellular route (between ECs) and by a trans-cellular route (directly through individual ECs). This diapedesis occurs through discrete pores and gaps in the endothelial monolayer mediated by VCAM-1-enriched trans-migratory cups [4]. Both lamellipodia (which are flattened cellular extensions on the cell surface) and invadosomes (which constitute a dot-like accumulation of filamentous actin at the site of cell–matrix contact) are involved in the trans-migratory process of MSCs [40]. However, unlike leukocytes, nonapoptotic membrane blebbing was also evident, as has been described in metastatic tumors [4]. Schmidt *et al.* observed that MSCs have a unique potency for abolishing, limbering, and splitting tight junctions between ECs during extravasation [41]. Using the MSC–EC co-culture system these authors showed that MSCs, after seeding on to an endothelial monolayer, became round and displayed cytoplasmic offshoots into the EC monolayer within 30 min, leading to the obliteration of tight junctions between ECs and the subsequent development of tight cell–cell contacts between MSCs and ECs with eventual trans-endothelial migration of MSCs. They also showed that connexin-43 had a key role in EC–MSC communication [41].

Chemokines are other adhesion-related molecules that are also involved in the trans-endothelial migration of MSCs (Figure 26.1B). These molecules are secreted in response to a number of signals, including proinflammatory cytokines, and are thought to bind glycosaminoglycans on ECs [42]. It is believed that sulfation of tyrosine residues in N-terminal regions of chemokine receptors occurs after interaction with their

corresponding ligands [43]. Chemokine receptors are G-protein-coupled receptors for CXC, CC, C, or CX3C chemokines. MSCs were found to express CCR1, CCR2, CCR4, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR2, CXCR4, CXCR5, CXCR6, and CX3CR1 receptors and to secrete a variety of chemokines [44–46]. It has been demonstrated that chemokine receptors and their ligands have an indispensable role in the migration of murine (m) MSCs across murine aortic ECs (MAECs) and that both chemokine stimulation and shear stress enhanced the trans-endothelial migration of mMSCs across MAECs [26]. At first, murine MSCs extended fine microvillous processes called filopodia and then extended pseudopodia in multiple directions. Subsequently, CXCL9, CXCL16, CCL20, and CCL25 ligands improved trans-endothelial migration across MAECs and shear forces markedly stimulated the crawling and spreading of the MSCs [26]. Fractalkine (CX3CL1) and its receptor CX3CR1, concomitant with the SDF-1/CXCR4 axis, governed the trafficking of transplanted MSCs into brain injuries [44] (Figure 26.1B). Flow cytometry analysis of brain biopsy samples from rats that had received an intracerebral injection of SDF-1α indicated an enhanced accumulation of 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFDA-SE)-labeled MSCs in the cerebral cortex [44]. Simultaneously, a significant increase in CXCR4 expression was evident by reverse transcription polymerase chain reaction analysis [44].

The ECM is a highly dynamic structure with an intricate network of proteins and serves a multitude of functions of biological significance, including the reciprocal interactions of cells with individual molecules during cell attachment, growth, migration, and apoptosis, as well as providing structural support of cells [47,48]. It has been documented that each ECM component imposes a battery of specific biological effects on MSCs. For example, fibronectin facilitates migration, adhesion, and proliferation, whereas fibrinogen augments adhesion and proliferation, but not migration [49] (Figure 26.1B). Xiao *et al.* showed that interaction between $\alpha_2\beta_1$ on MSCs and the collagen component of ECM instigated osteoblastic differentiation of MSCs via the MAPK pathway [50]. Interestingly, the integrin expression pattern of MSCs is related to specific cell behavior on specific ECM components [49]. In addition to direct MSC–EC crosstalk via receptor–ligand interactions, ECM also directs MSC migration during vascular remodeling [13].

In an *in vitro* model the epidermal growth factor (EGF)/EGF receptor axis and relevant downstream signaling pathways such as ERK, Akt, and FAK phosphoproteins also increased MSC migration [51]. MSCs also participate in an electrotaxis process and respond to endogenous electric fields, which occur naturally in healing wounds, especially bone injuries [52].

In addition to the role of ECs in the extravasation of MSCs, MSCs in turn contribute to the growth of ECs by developing EC–MSC islets via the secretion of proteases [53]. During myocardial infarction and in other inflammatory conditions, endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO) production, and subsequent PI3K/protein kinase B (Akt)/eNOS downstream signaling and MMP-9 activation triggered MSC as well as endothelial progenitor cell (EPC) migration to target tissues [54].

## 26.3 Mesenchymal stromal cell–endothelial cell crosstalk in angiogenesis

Various types of MSC–EC three-dimensional (3D) co-culture systems and *in vivo* models have revealed extensive cellular crosstalk during endothelial angiogenesis through a variety of mechanisms. This process is exquisitely controlled by cell–cell connections, paracrine and juxtacrine interactions, or microvesicle trafficking between the MSC and EC lineages, leading to modulation of the angiogenic response [2,55–58] (Figure 26.2).

### 26.3.1 Juxtacrine interactions of mesenchymal stromal cells and endothelial cells

In an *in vitro* Matrigel assay using rat and human MSC–EC co-culture systems, self-assembled and elongated tube-like structures were developed by intimate MSC–EC contact soon after seeding on Matrigel. Subsequently, an endothelial phenotype was induced in co-aligned MSCs via VEGF-dependent signaling and then activation of the Rho/ROCK pathway [57,59] (Figure 26.2). In this context, Rho, as a molecular switch, transmits signals from cell surface receptors to its effector protein ROCK, a Rho-associated kinase enzyme, which in turn contributes to the phosphorylation of LIM-kinase and the actin-regulatory protein Cofilin, eventually leading to the rearrangement of the actin cytoskeleton [60,61]. Blocking the RhoA/ROCK pathway by its relevant inhibitor, Y-27632, abrogated VEGF-mediated activation of ECs, disrupted tube formation on Matrigel, and reduced CD31 expression in ECs [62]. Intriguingly, the key modulatory effect of the RhoA/ROCK pathway in MSC orientation, elongation, alignment, and contact guidance was also determined on a patterned nanometric surface [63]. A number of studies have shown a significant upregulation in Rho activity during the development of a new cell adhesion, whereas its activity is downregulated in stably adhered cells [64]. Additionally, ROCK activity was shown to play a role during transmigration of MSCs from the brain endothelial barrier [65].

A pericyte-like function has been reported for MSCs *in vitro* and *in vivo* conditions during vascular network formation and stabilization through direct contact with ECs [66–68]. The SLIT3/ROBO4 (roundabout homolog 4, also known as magic roundabout) signaling pathway, as also described in pericytes, was activated when MSCs were juxtaposed and co-aligned with ECs in a co-culture system [69] (Figure 26.2). It has been reported that downregulation of VEGF receptor (VEGFR) occurred immediately after activation of ROBO4 [70]. Seeding of human ECs and MSCs on to poly(L-lactide-co-1,5-dioxepan-2-one) scaffold in a 5 : 1 ratio increased Ang-1 and perivascular gene

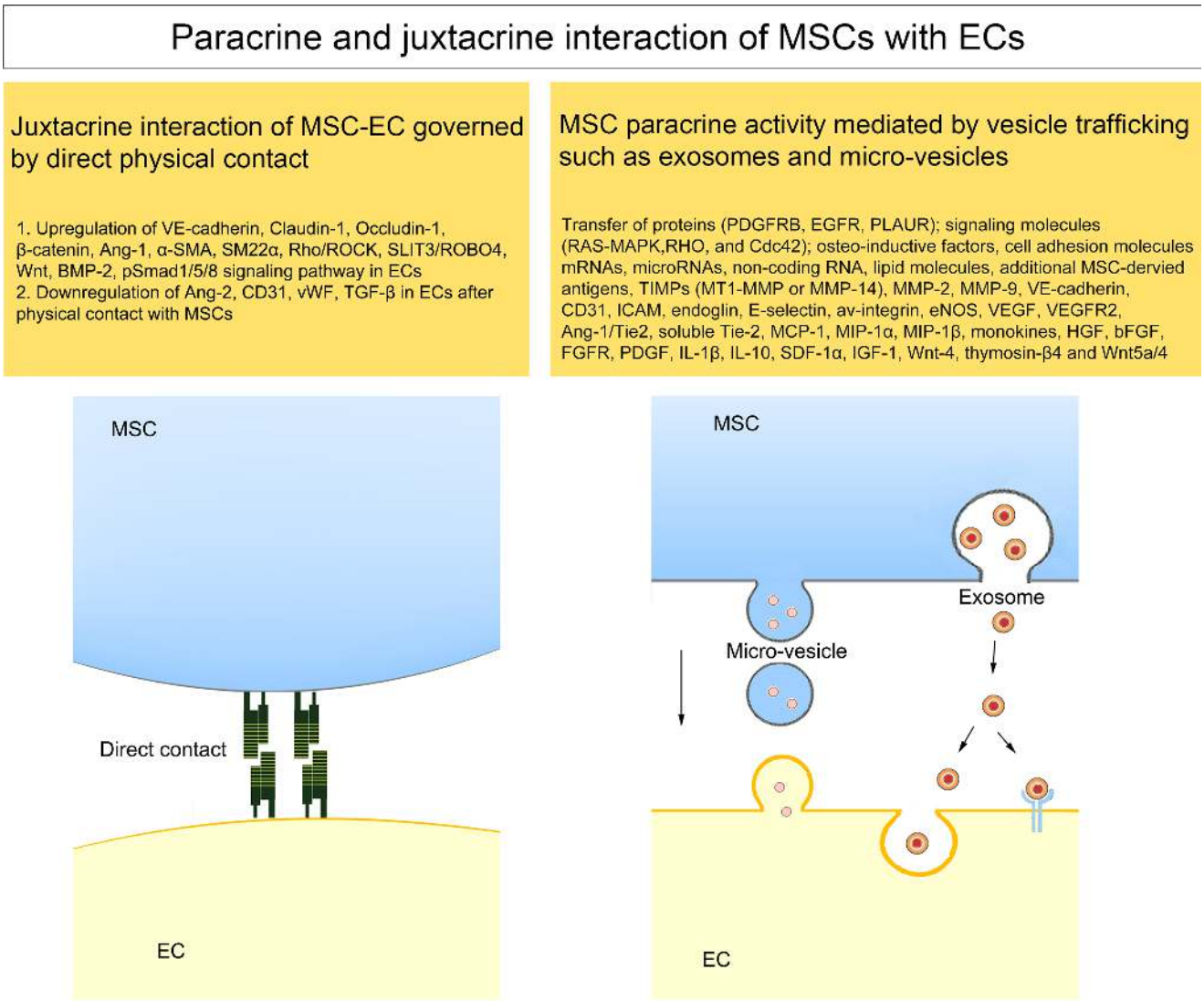



**Figure 26.2** MSC–EC interactions occur via different juxtacrine and paracrine mechanisms. A variety of signaling molecules participate in juxtacrine interaction between MSCs and ECs. In addition, many molecules are transferred between MSCs and ECs in a paracrine manner through vesicle trafficking, such as exosomes and microvesicles. Ang: angiopoietin; bFGF: basic fibroblast growth factor; Cdc42: cell division control 42; EGFR: endothelial growth factor receptor; FGFR: fibroblast growth factor receptor; HGF: hepatocyte growth factor; MCP-1: monocyte chemoattractant protein-1; MIP: macrophage inflammatory protein; PDGFR: platelet-derived growth factor receptor; PLAUR: urokinase-type plasminogen activator receptor; Tie-2: tyrosine kinase with immunoglobulin-like and EGF-like domains 2; TIMPs: tissue inhibitors of MMPs; VEGF: vascular endothelial growth factor; VEGF receptor (VEGFR)2.

expression, such as α-smooth muscle actin (α-SMA) and SM22α, whereas a pronounced reduction in Ang-2, CD31, and vWF was apparent [71]. Moreover, MSCs that expressed stemness markers, such as CD146, Sca-1, and PDGFRα, were identified in close proximity to vessels with a propensity to express the pericyte-like markers NG2, CD146, and PDGFRβ. It has been demonstrated that $CD146^+$ pericytes represent a subpopulation of bone marrow MSCs that supports neoangiogenesis [72]. *In vivo* perivascular homing of MSCs along the vessel wall was observed after intravenous injection of MSCs in an ischemic murine skin flap model [73]. *In vivo* co-implantation of MSCs isolated from four murine tissues – bone marrow, white adipose tissue, skeletal muscle, and myocardium – with human blood-derived endothelial colony-forming cells led to the development of a complex network of blood vessels with ECs lining the lumens and MSCs present in the perivascular space immediately adjacent to the luminal structures [74]. Immunofluorescence assays by our group revealed the localization of green-fluorescent-protein-labeled rabbit bone-marrow-derived MSCs both in the lumen and in the wall of newly formed microvessels after injection of these cells into the border zone of infarcted myocardium [2]. The existence of MSCs in the peri-endothelial space highlights that the endothelium, as an important component of the MSC niche, can regulate the biological activity of MSCs [75–77].

MSC-derived spindle-shaped myofibroblastoid cells containing organized smooth muscle α-actin filaments were also detected in the EC–MSC co-culture [78]. With respect to intracellular signaling, HUVECs, in a 3D spheroid co-culture system, activated endogenous Wnt as well as bone morphogenetic protein (BMP) signaling in human MSCs with an increased level of nuclear β-catenin and pSmad1/5/8 under osteogenic conditions [79] (Figure 26.2). MSC–EC interaction resulted in the expression and co-localization of VE-cadherin and β-catenin at the cell membrane, vascular tube breakdown, and inhibition of endothelial permeability with the preservation of VE-cadherin, Claudin-1, and Occludin-1 in pulmonary ECs in a rat model of hemorrhagic shock-induced acute lung injury [80] as well as in an *in vitro* study using a human MSC–EC co-culture system [81,82] (Figure 26.2). As the soluble modulator of Wnt signaling, secreted frizzled-related protein (sFRP)-1 upregulated PDGF-BB in murine MSCs and facilitated β-catenin-dependent MSC–MSC, MSC–EC, and MSC–smooth muscle cell adhesions *in vitro* [83]. After subcutaneous injection of MSCs mixed with Matrigel in mice this pathway in turn triggered MSC glycogen-synthase-kinase-3β-dependent induction of angiogenesis along with vessel maturation and functionality, but not endothelial differentiation of MSCs [83,84]. Other studies have implicated sFRP-2 as a key molecule for the regenerative potential of murine MSCs following their transplantation into myocardial infarction tissue [85]. Likewise, specific knockdown of sFRP-2 in murine MSCs decreased the MSC engraftment and vascular density in the tissue [85].

The proliferation rate of rat MSCs was reportedly reduced in MSC–EC co-culture as a result of the downregulation of a number of growth factors, particularly in members of the TGF-β family [55]. Duffy *et al.*, however, showed that human MSCs increased aortic EC proliferation in a noncontact co-culture system [86]. Bidarra *et al.* reported that human MSC–EC co-culture not only augmented the proliferation of MSCs but also triggered osteogenic differentiation via ECs-dependent BMP-2 signaling [87]. These authors used three ratios of MSC : EC (3 : 1, 1 : 1, and 1 : 3) and found that the relative number of ECs declined over time in all cell ratios, with the highest percentage of MSC : EC ratio (3 : 1) resulting in the lowest relative number of ECs. However, when rat MSCs were added to rat lung microvascular EC-derived capillaries on Matrigel at EC : MSC ratios of 1 : 1 or 3 : 1, degeneration of the capillaries was evident due to the production of reactive oxygen species by MSCs [32]. Such discrepancies may be partly related to culture conditions and difference in the origin of cells [87]. Adding human MSCs at increasing doses from 1 : 10 to 1 : 1 MSC : EC ratio, in order to pre-establish a human aortic endothelial lattice on Matrigel, resulted in a proportional increase in the thickness of vessel-like structures as well as in the size of junctions. In contrast, the addition of MSCs prior to EC seeding on Matrigel resulted in EC bunching and loss of lattice formation [86]. Thus, the addition of MSCs to the fully formed endothelial lattice led to the stabilization and maturation of pre-established vessel-like structures, whereas MSCs prompted EC migration and bunching at early time points during vessel formation [86,87].

Overall, it appears that the inhibitory or stimulatory effect of MSCs on the behavior of ECs on Matrigel is dependent not only on the MSC : EC ratio in co-culture systems, but also on the sequence of cell addition in different co-culture experiments. Distinct signaling mechanisms can change cell–cell ratios. An example is during development of the thymus, where change in the intrathymic CD4 : CD8 cell ratio from the fetal to adult thymus occurred as a result of differential expression of Notch signaling molecules by thymic stromal cells [88]. A change in cell : cell ratios may also activate distinct signaling mechanisms, with subsequent development of different phenotypic and genotypic profiles.

### 26.3.2 Paracrine interactions of mesenchymal stromal cells and endothelial cells

MSCs have a dynamic effect on their microenvironment through production of a variety of biomolecules, including cytokines, chemokines, growth factors, and ECM molecules in a paracrine (on nearby cells) or autocrine (on the same cell that secreted the molecule) manner (Figure 26.2). Among this heterogeneous mixture of proteins, a variety of proangiogenic, immunosuppressive, anti-inflammatory, and antifibrotic factors together with ECM homeostasis regulators, such as several types of collagens, as well as TIMPs, have been detected [89].

Membrane-derived or extracellular vesicles are important mediators of intercellular communication essential for exchanging information and transmission of biological signals, and now designated the signalosome [90] (Figure 26.2). Three classes of extracellular vesicles have been described: exosomes, microvesicles, and apoptotic bodies [90] (and see Chapters 20 and 38). Exosomes and microvesicles are enclosed by a lipid bilayer and constructed from the inward budding of an endosomal membrane and from budding of the plasma membrane respectively [90,91]. The extracellular vesicles of MSCs, including microvesicles and exosomes (or nanovesicles), contain many proteins [92]. MSC-derived exosomes are 30–100 nm in diameter, express characteristic exosomal markers, such as CD9, CD63, and CD81 [93], and serve to transfer molecules, including proteins, transcription factors, small and large non-coding regulatory RNAs, such as mRNAs, microRNAs, and lipid molecules (Figure 26.2). While therapeutic effects of MSC-derived exosomes have been detected in cancer and in regenerative medicine, including cardiovascular and hepatic regeneration [93,94], information on the role of exosomes in many pathophysiological processes is in its infancy. However, MSC exosomes have some proangiogenic properties [93,94]. Among the proteins identified by proteome analysis of human MSC exosomes are PDGFRB, EGFR, and PLAUR as well as signaling molecules such as RAS-MAPK and RHO. Other proteins identified in the MSC exosome include Cdc42 cell adhesion molecule, osteoinductive factors, and some characteristic MSC surface markers [95,96] (Figure 26.2). A variety of factors dynamically regulate the rate of

exosome secretion by MSCs [97]. For example, under hypoxic conditions of 1% and 3% $O_2$, the rate of exosome secretion by placental MSCs was enhanced by 3.3- and 6.7-fold respectively, resulting in enhancement of the migration and angiogenic tube formation of placental microvascular ECs *in vitro* [97]. By contrast, in the mouse breast cancer cell line 4T1 the expression of the proangiogenic molecule VEGF was significantly downregulated by murine MSC-derived exosomes [98]. In addition to the dynamic interactions via exosomes of MSCs with cells of other lineages, ECs were also found to release extracellular vesicles in response to cellular activation or apoptosis [99,100]. In this context, different kinds of soluble factors, such as VE-cadherin, PECAM-1 (CD31), ICAM-1, endoglin, E-selectin, S-endo or $\alpha_v$ integrin, eNOS, and VEGFR2 were transferred by EC-derived extracellular vesicles [101,102] (Figure 26.2).

By virtue of their versatility, MSC-derived exosomes are now considered as a potential cell-free therapeutic approach that may avoid the some of the difficulties associated with cell therapy. A beneficial effect of MSC exosomes has been demonstrated in preclinical models of stroke [103,104], ischemic heart disease [105], acute lung injury [106], liver fibrosis [93], rheumatologic diseases [107], and central and peripheral nerve degeneration [108,109], as well as in a number of malignancies, including glioma [110] and multiple myeloma [111]. MSC-derived exosomes have also been utilized for horizontal drug cargo sorting and transfer to recipient cells [112].

Large numbers of soluble factors have been identified in MSC-derived conditioned medium. These include VEGF, VEGFR2, Ang-1/Tie-2, MCP-1, MIP-1α, MIP-1β, fibroblast growth factor-2 (FGF-2), PDGF, IL-1β, IL-10, HGF, SDF-1, IGF-1, thymosin-β4, and Wnt5a [57,89]. MSC-derived VEGF can act as a stimulus for the survival and functional commitment of ECs [113]. In addition, a soluble Tie-2 receptor fragment generated by ectodomain proteolytic cleavage [114] was detected in rat MSC-conditioned medium, which appeared to be involved in the angiogenic axis [57].

The elimination of Wnt-4 signaling in murine MSCs preconditioned with hypoxia abolished the vasculogenic properties of these cells in a mouse ischemic hind limb model [115], suggesting that the Wnt signaling pathway possesses a critical role in the paracrine proangiogenic properties of MSCs. Overexpression of Wnt-4 induced osteogenic differentiation of human MSCs via the p38 MAPK in a novel noncanonical signaling pathway [116]. Induction of canonical Wnt signaling resulted in upregulation of the Wnt target gene Cyclin D1, which in turn led to an enhanced proliferative potential of murine MSCs [117]. Wnt-3a stimulation contributed to enhanced downregulation of MMP-2 and MMP-9 in MSCs, whereas Wnt signaling inhibition, by knockdown of the transcriptional activator β-catenin, diminished membrane type 1-matrix metalloproteinase (MT1-MMP or MMP-14), highlighting the regulatory role of Wnt signaling in MSC invasion and recruitment [117].

Dynamic mechanical signal transduction by compressive loading has also been considered to have an effect on gene expression and function of MSCs [118]. Stretch-activated ion channels, cell adhesion molecules with activation of second messenger pathways, and downstream signaling cascades such as growth factor tyrosine kinases, G protein-coupled receptors, p21ras, MAPK, ribosomal S6 kinases (pp90RSK), protein kinase C, phospholipase C, and phospholipase D participated in the transduction of mechanical stimuli from the external environment to the interior of the cells [119,120]. Interestingly, both autocrine and paracrine mechanisms were shown to be involved in such stretch responses, since stretch-conditioned medium mimicked the effect of stretch on nonstretched cells, especially cardiomyocytes [119]. Furthermore, in a bioreactor system for modeling the fracture gap, mechanical loading induced the upregulation of a battery of proangiogenic molecules, such as FGF-2, FGF receptor, VEGFR, TGF-β, MMP-2, MMP-9, and MT1-MMP (or MMP-14) in human MSCs during the early phase of bone healing [120,121]. In general, it can be concluded from this set of data that milieu-dependent conditions, including cell–cell contact, soluble paracrine factors, and mechanical stimulation, ultimately determine the biological activity of MSCs in physiological and pathological conditions.

## 26.4 Mesenchymal stromal cell–endothelial cell crosstalk in tumor angiogenesis

Tumor angiogenesis is a critical component of tumor progression and metastasis. It is under the control of a number of endogenous and exogenous factors that influence the tumor microenvironment. There is controversy on the role of MSCs in tumor angiogenesis, with both stimulatory and inhibitory effects having been reported for tumor neoangiogenesis [56].

### 26.4.1 Stimulation

Subcutaneous co-injection of MSCs and c26 colon cancer cells in mice resulted in the development of larger tumors with enhanced cancer angiogenesis compared with the injection of the cell line alone. This growth-promoting effect was further enhanced after pretreatment of MSCs with inflammatory factors IFN-γ and TNF-α [122]. Enhanced angiogenesis in this system was achieved by upregulation of VEGF through activation of the hypoxia-inducible factor (HIF)-1α signaling pathway in MSCs. In another study, co-transplantation of the mouse breast cancer cell line 4T1 and murine MSCs, or co-injection of the human prostate cancer cell line DU145 and human MSCs, into nude mice led to increased tumor size with a greater vascular area [123]. Increased expression of the proangiogenic factors TGF-β, VEGF, IL-6, and MIP-2 was detected in human MSCs exposed to DU145-conditioned medium or co-cultured with DU145 (Figure 26.3).

Co-cultures of murine MSCs and B16 melanoma cells also resulted in secretion of higher concentrations of the cytokines LIF, M-CSF, MIP-2, and VEGF [124]. Moreover, co-transplantation of MSCs and B16 melanoma cells or Lewis lung carcinoma cells into

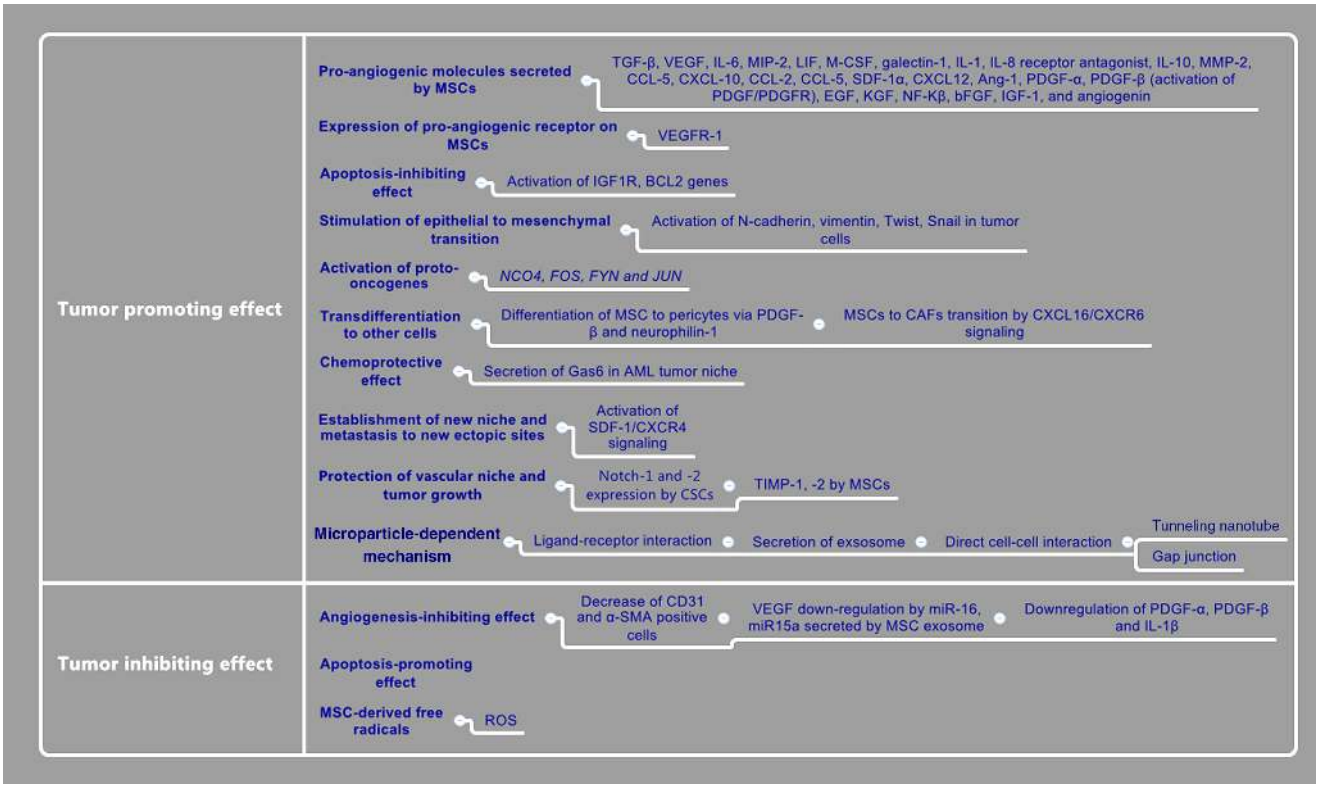



**Figure 26.3** Effects of MSCs on the biology of tumors. MSCs exert a large number of both stimulatory and inhibitory effects on tumor development and proliferation through multiple mechanisms. KGF: keratinocyte growth factor; LIF: leukemia inhibitory factor; M-CSF: macrophage colony-stimulating factor; NF-κB: nuclear factor kappa light chain-enhancer of activated B cells; ROS: reactive oxygen species.

mice led to increased tumor size with enhanced vessel density [124]. The transplanted MSCs were tracked near tumor vessels and attained a $CD31^+$ endothelial-like phenotype. MSC-secreted IL-6 and VEGF were shown to be paracrine migratory stimuli for breast cancer cells [125]. MSCs can also induce changes in the transcriptome of tumor cells. Direct co-culture of human MSCs with a number of breast cancer cell lines, including T47D, SK-Br3, and MDA-MB-231, resulted in upregulation of the proangiogenic gene *VEGF* and the antiapoptotic genes insulin-like growth factor 1 receptor (*IGF-1R*) and B cell lymphoma (*BCL2*) in breast cancer cells, as well as overexpression of the oncogenes nuclear receptor coactivator 4 (NCOA4) and FOS (see Glossary), the proto-oncogenes *FYN* and *JUN*, and genes involved in epithelial-to-mesenchymal transition (EMT), such as N-cadherin, vimentin, Twist, and Snail. These findings suggest that MSCs may act as a trigger to induce EMT in breast cancer cells and actively promote breast cancer metastasis [126] (Figure 26.3). Tumor-cell-derived proangiogenic factors may induce MSCs to take on an endothelial-like morphology. When MSCs were seeded on Matrigel in an indirect transwell co-culture with the MCF-7 breast cancer cell line, breast cancer cells stimulated MSCs to organize into capillary-like structures, which was similar to those observed when MSCs were cultured in the presence of VEGF [127].

It appears that galectin-1, an immunosuppressive and proangiogenic member of the β-galactoside-binding lectin family, is involved in regulation of tumor progression and angiogenesis by MSCs (Figure 26.3). Compared with control wild-type MSCs, orthotopic co-injection of galectin-1-deficient MSCs and 4T1 cells resulted in a reduction of tumor growth and development [128]. Consistent with these findings, it was demonstrated that galectin-1 stimulated the proliferation of B16F10 melanoma cells through a marked decrease in the expression of p53/p21, indicating that galectin-1 antagonists may be a useful therapeutic target in melanomas [129].

Tumor-cell-derived paracrine mechanisms play a key role in the fate of MSCs in the tumor. Conditioned media from different tumor cell lines directed MSCs to differentiate into mature pericytes by tumor-cell-secreted PDGF-B with recruitment of MSC-derived pericytes to the newly formed capillaries *in vitro* and *in vivo* [130]. In this context, conditioned media from more aggressive tumor cells had less abundant recruitment of pericytes, with potential correlation with metastatic spread of these cells. Tumor PDGF-B may induce mesenchymal to pericyte transition through an interaction with neuropilin-1 in MSCs [130] (Figure 26.3). In a murine model of vascular injury it was shown that interaction between CX3CR1 and fractalkine resulted in smooth muscle cell differentiation of $CX3CR1^+$ bone marrow progenitor cells at the site of injury [131].

The secretion of the proinflammatory peptide LL-37 (leucine, leucine-37), the C-terminal peptide of human cationic

antimicrobial protein 18, in a variety of carcinomas, including ovarian carcinoma, can induce MSCs to migrate into the tumor mass and augment MSC secretion of a number of cytokines and proangiogenic factors, including IL-1 receptor antagonist, IL-6, IL-10, CCL5 (also known as RANTES, regulated upon activation, normal T-cell expressed, and secreted), VEGF, and MMP-2, resulting in MSC-induced tumor progression [132] (Figure 26.3). HIFs were also shown to act as mediators of bidirectional paracrine signaling between MSCs and breast cancer cells [133]. HIF-dependent interaction between MSCs and breast cancer cells resulted in expression of CXCL10 and CCL5 in MSCs and their cognate receptors CXCR3 and CCR5 in breast cancer cells, as well as expression of placental growth factor and its cognate receptor VEGFR1 by breast cancer cells and MSCs respectively. This led to the expression of genes that promote invasion and metastasis of breast cancer cells.

Local and recruited MSCs also participate in the tumor niche through differentiation into cancer-associated fibroblasts (CAFs) with a capability of developing cytokine networks that promote tumor growth, progression, and metastasis and create chemotherapy resistance in many solid tumors, including brain cancer, breast cancer, and colon cancer [134–138]. In prostate cancer it has been shown that the expression of the CXC chemokine ligand 16 (CXCL16) by tumor cells resulted in the recruitment of bone-marrow-derived MSCs through interaction with its receptor CXCR6, which is expressed by marrow MSCs [139]. The CXCL16/CXCR6 signaling induced the conversion of MSCs into CAFs with subsequent secretion of a high level of SDF-1 (also known as CXCL12) (Figure 26.3). SDF-1 expression by CAFs promoted EMT of the prostate cancer cells and augmented the expression of its receptor CXCR4, resulting in enhanced tumor progression, extravasation, and metastasis [139]. Thus, MSCs and the CXCL16/CXCR6 axis may be a potential therapeutic strategy for curbing tumor progression and metastasis in prostate cancer. Activation of the PDGF/PDGFR axis through interactions of PDGF-expressing gastric and colon tumor cells and PDGFR-expressing CAFs was shown to play a critical role in the growth and metastasis of gastric and colon cancers in orthotopic transplantation models [140,141]. In a study of orthotopic transplantation and splenic injection of human MSCs together with KM12SM human colon cancer cells, blockade of PDGFR signaling with imatinib, which is a potent tyrosine kinase inhibitor of PDGFR, inhibited the tumor-promoting effect of MSC-derived CAFs and significantly reduced the migration and survival of MSCs in the tumor microenvironment [142].

MSCs from different tissues may have different biological effects on tumor growth and angiogenesis. While co-culture of glioma cells with human umbilical-cord-blood-derived MSCs resulted in inhibition of tumor growth and induction of apoptosis in tumor cells via TNF-related apoptosis-inducing ligand (TRAIL), adipose-tissue-derived MSCs promoted glioma growth, which was associated with the overexpression of the proangiogenic factors VEGF, Ang-1, PDGF-α, PDGF-β, IGF, and SDF-1 [143] (Figure 26.3). Because of the inhibition of TRAIL activation of the apoptotic pathway by SDF-1, the authors concluded that adipose-tissue-derived MSCs supported glioma development by both promotion of angiogenesis and inhibition of apoptosis.

### 26.4.2 Inhibition

It seems that the role of MSCs in the tumor niche is determined based on a spatiotemporally dependent process. When human MSCs were administered systemically to nude mice bearing tumors produced by the injection of TSA-pGL3 mouse adenocarcinoma cells or injected directly in contact with pre-established tumors, a significant reduction in tumor growth was achieved due to an inhibition of tumor cell proliferation [144]. The authors showed a significant decrease in the number of $CD31^+$ tumor vessels and increases in vessel length and in the percentage of $\alpha$-$SMA^+$ cells after MSC administration, indicating that MSCs may be useful for the treatment of established tumors and might be able to induce vascular remodeling in them [144] (Figure 26.3).

MSC-derived exosomes were also effective in the tumor niche through downregulation of the expression of VEGF in breast cancer cells, leading to inhibition of angiogenesis *in vitro* and *in vivo*, which may be in part mediated by miR-16, a microRNA known to target VEGF in exosomes [98] (Figure 26.3). The macromolecular content of MSC exosomes appears to differ in a context-dependent manner. A lower level of the tumor suppressor miR-15a and a higher level of CCL2 (also known as MCP-1) were detected in exosomes derived from bone marrow MSCs from people with myeloma compared to those from normal controls, suggesting that exosomes from myeloma MSCs promoted tumor growth in contrast to exosomes from normal bone-marrow-derived MSCs [111] (Figure 26.3).

Co-transplantation of human bone marrow MSCs and glioma cells into immunodeficient mice resulted in a significant reduction in tumor volume and microvessel density. The antiangiogenic property of MSCs was ascribed to reduced expression of PDGF-BB, PDGFR-β, and IL-1β [145]. The antitumor effect of MSCs in this model might have been due to suppression of tumor angiogenesis though the downregulation of the PDGF/PDGFR axis (Figure 26.3). Upregulation of the tumor suppressor phosphatase and tensin homolog (PTEN) with subsequent downregulation of Akt and PI3K signaling pathways was induced in glioma cells after co-culture with human umbilical-cord-blood-derived MSCs and glioma cells [146].

## 26.5 Endothelial differentiation of mesenchymal stromal cells

Transdifferentiation of MSCs into multiple mesodermal lineages, including differentiation into endothelial-like cells, has been demonstrated using *in vivo* and *in vitro* systems [147–151] (Figure 26.4).

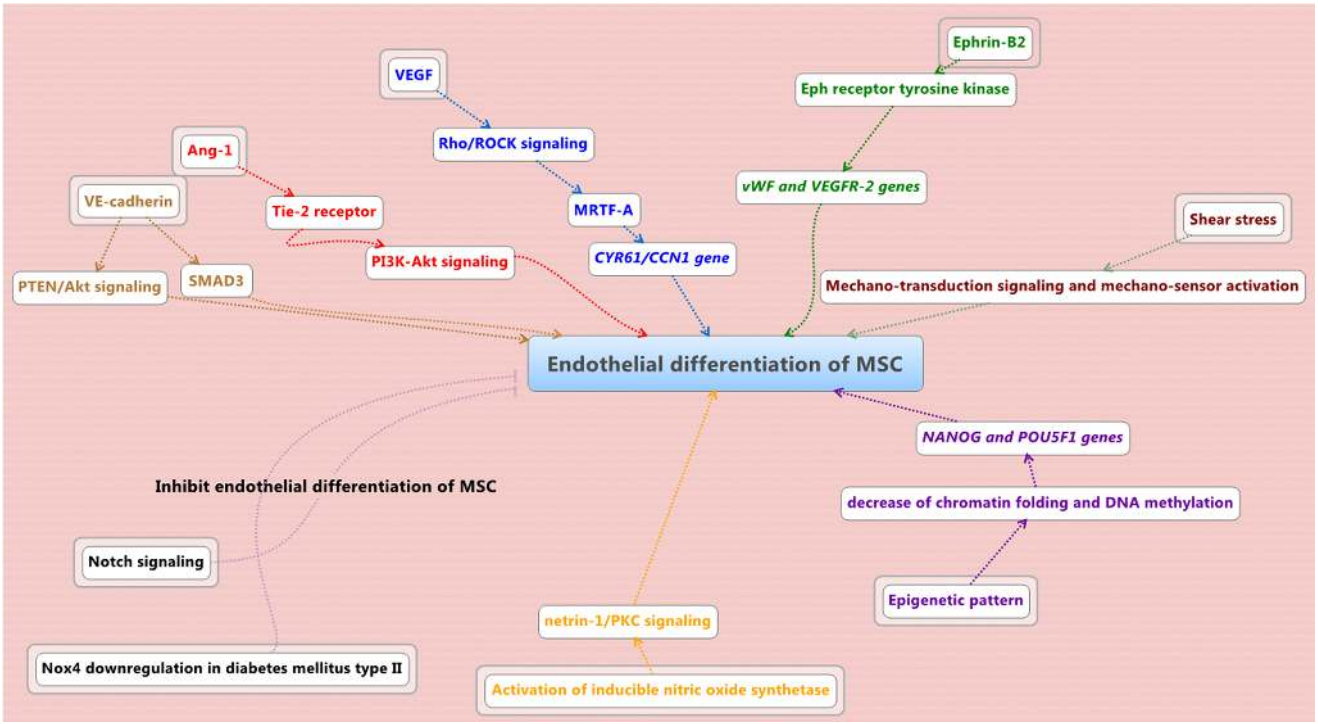



**Figure 26.4** Endothelial differentiation of MSCs and the switching on of EC-specific genes in MSCs is controlled by different signaling pathways. Multiple signaling pathways in addition to mechanical stimulation may trigger differentiation of MSCs into ECs.

However, the exact mechanisms mediating the endothelial differentiation of MSCs including stimulatory or inhibitory factors, intracellular signaling molecules or extracellular nano-mechanical signals is still being determined [152,153]. Because of the importance of neoangiogenesis in regenerative medicine together with the potential of MSCs to repair damaged tissues of the musculoskeletal system, many attempts have been made to use MSCs both as a source of cells for such tissue repair as well as precursors of ECs [56,149,154]. There is debate, however, with regard to the endothelial differentiation of MSCs. Issues include culture conditions, the tissue microenvironment of the recipient, and subpopulations of MSCs [153].

Differentiation of MSCs into endothelial-like cells has been associated with the expression of EC-specific markers on MSCs – including kinase insert domain receptor (KDR – a type III receptor tyrosine kinase also known as VEGFR2), FMS-like tyrosine kinase 1 (FLT-1), vWF, VEGFR1, endothelial differentiation-related factor 1, angio-associated, migratory cell protein, CD31, CD34, and cadherin 5, type 2 (vascular endothelium) (CDH5) – together with integrins such as ICAM-2 and down-regulation of stemness markers, including CD9, E-cadherin, podocalyxin-like protein, and stage-specific embryonic antigen-4. This has resulted in an extensive change in the behavior of these cells toward endothelial-like cells [147,155–157]. However, König *et al.* failed to detect amnion-derived MSCs with complete differentiation to mature ECs because of downregulation of proangiogenic factors such as tenascin C, Tie-2, VEGF, and FGF2 and upregulation of the antiangiogenic factors serpin F1, sprouty1, angioarrestin, and endostatin [154]. Furthermore, some *in vitro* experiments have cast doubts on the endothelial differentiation potential of some tissue-specific MSCs; for example, Wharton's jelly MSCs, with which *in vitro* cell manipulation or passage number altered the expression of EC-related markers, such as CD31, CD34, vWF, VE-cadherin (CD144), Tie-2, VEGFR-1, and VEGFR-2 [158,159]. Alaminos *et al.* also reported incomplete transdifferentiation of Wharton's jelly MSCs into ECs with no or only partial induction of genes related to endothelial function, including *CD36* (fatty acid translocase), *ECE2* (endothelin-converting enzyme), *vWF*, *THBD* (Thrombomodulin), *PGI2* (proliferation-inducing gene), *ECE1*, and *ACE* (angiotensin-converting enzyme) [157].

Conversely, a number of *in vitro* studies using specific experimental conditions, such as hypoxia or osteogenic differentiation, induced MSCs to secrete VEGF and its receptor VEGFR1 (FLT-1) [160]. Moreover, the potency of MSCs showing an endothelial-like phenotype has been confirmed in a number of studies in which MSCs were used for regenerative purposes [2,161]. We demonstrated the contribution of $CD31^+$ MSCs to the structure of myocardial capillaries as early as 7 days after their transplantation into infarcted myocardium [2].

As alternative inducers of differentiation, extracellular mechanical signals can actively direct EC differentiation of MSCs at both genotypic and phenotypic levels. In this context, the interaction of ECM proteins with MSC surface receptors and subsequent induction of intracellular mechano-transduction signaling resulted in endothelial differentiation of MSCs [153,162].

Mechano-sensors and mechano-transduction signaling pathways also support the fate of MSCs to an endothelial-like phenotype under shear stress [163] (Figure 26.4). A steady exposure of canine bone-marrow-derived MSCs to shear stress in a pulsatile bioreactor promoted MSC differentiation into endothelial-like cells after 4 days [164]. The authors found a profound increase in CD31, VE-cadherin, and CD34 at both mRNA and protein levels, while protein levels of α-SMA and calponin were reduced. Concurrently there was a change in cell morphology from a fibroblast-like appearance to an elongated fusiform shape in MSCs seeded onto poly-ε-caprolactone and lactic acid scaffolds [164]. In addition to soluble induction factors that promoted initial lineage differentiation of MSCs, ECM stiffness or elasticity has also been shown to be involved in commitment of MSCs to a specific lineage [165,166]. As an example, microarray profiling of MSC transcripts in cells cultured under 0.1, 1, 11, or 34 kPa matrices yielded neurogenic markers at 0.1–1 kPa, while myogenic and osteogenic markers were increased at 11 kPa and 34 kPa pressures respectively [165].

It has been demonstrated that adaptation of MSCs to a specific shape plays an important role in the fate of these cells [167]. Mahmoudi *et al.* demonstrated that the imprinted topography of silicon-made templates altered the gene expression profile of rabbit adipose-tissue-derived MSCs, while the imprinted patterns of mature chondrocytes induced both collagen type II and aggrecan expression in these cells. The imprinted pattern of spindle-shaped fibroblast-like cells did not induce collagen II expression and decreased aggrecan expression [167].

Numerous factors and their downstream signaling pathways have been reported to orchestrate MSC differentiation into EC-like cells. Both human and rat MSC differentiation to endothelial-like cells was achieved via the VEGF Rho/myocardin-related transcription factor-A (MRTF-A) signaling pathway [59] (Figure 24.4). It was shown that the activation of the Rho/ROCK signaling pathway promoted the nuclear translocation of MRTF-A, and knockdown of MRTF-A selectively reduced VEGF-derived differentiation of MSCs into endothelial-like cells [59]. VEGF can upregulate the promoter activity of cysteine-rich angiogenic inducer 61 (CYR61), a regulator of vascular development and angiogenesis. MRTF-A knockdown resulted in reduction of the VEGF-induced activation of the CYR61 promoter [59] (Figure 26.4). In contrast, it was found that upregulation of the myocardin transcription factor prompted MSCs to differentiate into smooth muscle cells and thwarted the differentiation of human MSCs into other lineages, such as ECs [168,169]. VEGF inhibited the non-EC differentiation of rat MSCs. As an example, VEGF hampered osteogenic differentiation of MSCs via BMP2 mRNA reduction *in vitro* [170]. Conversely, genetic modification of bone marrow MSCs via adenoviral delivery of Ang-1 accelerated both epidermal and dermal reconstitution by induction of an EC-like phenotype and increased CD31 expression [171]. Ang-1 had the strongest influence on endothelial differentiation of stem cells, especially embryonic stem cells, via activation of Tie2–PI3K–Akt as a major downstream signaling pathway responsible for Ang-1-induced $CD31^+/CD144^+$ EC expansion [172] (Figure 26.4). SMAD3, a downstream effector of TGF-β2 and inhibition of the PTEN/Akt pathway, participated actively in differentiation into EC-like cells from induced pluripotent stem cells, and this was associated with upregulation of VE-cadherin [173] (Figure 26.4).

In addition to extrinsic factors, distinct epigenetic patterns, including chromatin folding and DNA methylation, can change the expression of some genes, which ultimately contributes to cell reprogramming and phenotypic change [174]. Culmes *et al.* pretreated human adipose-tissue-derived MSCs for 48 h with the epigenetic drug BIX-01294 (BIX), a novel methyltransferase and a histone G9a inhibitor, with subsequent differentiation in endothelial inductive medium for 7 and 14 days [175]. By this method, a pronounced decrease of total DNA methylation along with increased expression of pluripotency-related genes *POU5F1* and *NANOG* was achieved, which was associated with subsequent improvement in the differentiation capacity of MSCs into ECs [175] (Figure 26.4).

It is well established that NO signaling has an important role in the endothelial differentiation and maturation of EPCs [176,177]. In this context there is some evidence that NO signaling participates in the differentiation of MSCs to ECs, although the precise mechanisms behind the proangiogenic effects of NO require further clarification [178]. The homozygous knockout of *S*-nitrosoglutathione reductase (GSNOR) gene ($GSNOR^{-/-}$), a denitrosylase that regulates *S*-nitrosylation in murine MSCs, strongly inhibited the vasculogenesis potential of these cells both in an *in vitro* Matrigel tube-formation assay and in an *in vivo* Matrigel plug assay performed in immunocompromised mice. This was consistent with NO/GSNOR imbalance [178]. The activation of the inducible NO synthase/netrin-1/PKC signaling pathway in rat MSCs that had been subjected to reprogramming with Sonic Hedgehog (*SHH*) resulted in enhanced viability and angiogenic properties of these cells in the heart after myocardial infarction [179]. In addition to the NO-derived angiogenic activity of MSCs, NO-mediated osteogenic differentiation was obtained in physically activated rat marrow MSCs *in vitro* [180]. Moreover, the mechanical stimulation of MSCs also promoted both NO and prostaglandin $E_2$ release, which was associated with osteogenic differentiation [181]. It was also shown that prostaglandin $E_2$ secretion by transplanted MSCs in mice induced a switch from a proinflammatory phenotype to an anti-inflammatory phenotypic in macrophages, which was followed by the recruitment of EPCs and osteogenesis-promoting cells [182]. Molecular crosstalk has been reported between osteogenesis and angiogenesis in the skeletal system [183,184]. Notch signaling exerted an inductive effect for the release of Noggin from bone ECs, which was required for normal osteogenesis. In addition to impaired bone vessel morphology and growth, disruption of Notch signaling resulted in a reduction of osteogenesis, shortening of long bones, chondrocyte defects, and loss of bone trabeculae [184].

Overexpression of Ephrin-B2, a transmembrane ligand for Eph receptor tyrosine kinases, in human MSCs also induced an early EC-like phenotype with upregulation of vWF and VEGFR-2 (KDR/Flk-1) under EC culture conditions [86,185] (Figure 26.4). In contrast, Notch signaling induced smooth muscle cell markers in human MSCs as well as in human embryonic stem cells together with a decrease in endothelial markers in embryonic stem cells [185] (Figure 26.4). Activation of Notch-1 signaling under oxidative stress was shown to promote cardiogenic differentiation of rat MSCs. Yan and colleagues also demonstrated that oxidant stress, induced in type 2 diabetes mellitus restricted the differentiation of murine MSCs to EC-like cells by downregulation of Nox4 expression [186] (Figure 26.4).

## 26.6 Development of a biologically active niche through bidirectional endothelial cell–stromal cell crosstalk

Crosstalk between MSCs and ECs influences the tissue microenvironment (the niche) in various physiologic and pathologic conditions. Physiologically normal lung stem cells (LSCs) recruited ECs by a mechanism involving fibroblasts [187] (Figure 26.5A). A basal level of SDF-1 was released by LSCs that resulted in the activation of lung fibroblasts, leading to the production of paracrine signals, including TNF-α. This cytokine induced a positive feedback loop in LSCs, which ultimately enhanced the expression of SDF-1, thereby promoting the recruitment and further activation of fibroblasts. Lung fibroblasts activated by high levels of SDF-1 then expressed the angiogenic factors VEGF and IL-8, leading to the recruitment of ECs that created the functional microenvironment required for normal self-renewal and differentiation of LSCs into bronchiolar and alveolar lineage cells (see Figure 26.5A). MSCs and ECs also constitute major components of the tissue microenvironment in pathologic conditions. MSC–EC crosstalk plays an important role in the development of the tumor niche [188], which allows maintenance and expansion of the tumor by protection of cancer stem cells (CSCs). Thus, the tumor niche may be a target for novel therapies [189,190]. For example, a number of acute myeloid leukemia (AML) cells express the tyrosine kinase receptor Axl and are able to induce expression and secretion of the Axl ligand growth-arrest-specific gene 6 (*GAS6*) by marrow MSCs in the tumor niche [191]. Gas6–Axl paracrine interaction between AML cells and MSCs creates a chemoprotective tumor niche, resulting in proliferation, survival, and chemoresistance of Axl-expressing AML cells (Figure 26.3). Targeting this tumor niche through Axl inhibition is a potential therapeutic strategy for AML. Furthermore, development of a new niche is a prerequisite for the dissemination of neoplastic cells in many tumor types. An example is the establishment of the SDF-1/CXCR4 axis through interaction of $CXCR4^+$ acute lymphoblastic leukemia (ALL) cells and $SDF\text{-}1^+$ bile duct epithelial cells, which provides a hepatic niche that supports the extramedullary dissemination of ALL cells to the liver. Inhibition of the SDF-1/CXCR4 axis may be another novel therapeutic approach to ALL [192] (Figure 26.5B). It has also been demonstrated that the expression of SDF-1 by liver sinusoidal ECs and establishment of the SDF-1/CXCR4 axis created an extramedullary vascular niche for hematopoietic stem cells (HSCs), regulating their trafficking and homing of HSCs during extramedullary hematopoiesis in the liver [193]. The SDF-1/CXCR4 axis was also used by murine lymphatic ECs to develop a metastatic lymphatic niche in organs such as lymph nodes and lung. This facilitated the dissemination of $CXCR4^+$/$CD133^+$ progenitor cells in malignant melanomas [194] (Figure 26.3). SDF-1 may be expressed by lymph node stromal cells to support the formation of a microenvironment for lymph node metastasis [195]. In addition, the SDF-1/CXCR4 signaling is involved in the homing and localization of neural progenitor cells (NPCs) in the vascular niche of the adult subventricular zone in the brain [196] (Figure 26.3). The properties of the vascular niche seem to be context dependent. When subventricular zone NPC-derived neurospheres were cultured with intact or oxygen–glucose-deprived ECs, the normal ECs increased self-renewal of the NPCs and maintained these cells in their stem-cell-like state, whereas deprived ECs prompted migration and differentiation of neural stem/progenitor cells to serve as an endogenous repair mechanism for neuronal replacement after injury [197] (Figure 26.5C).

As indicated earlier, ECs provide a protective vascular niche for cancer stem cells (CSC) [198]. In the highly aggressive brain tumor glioblastoma multiforme, elimination of the EC niche resulted in a decrease in the self-renewal of tumor stem cells as well as a reduction in neurosphere-forming ability of neoplastic cells. This was due to the loss of EC-mediated CSC maintenance, which was mediated in part by Notch signaling [199]. Juxtacrine (close proximity) signaling between tumor cells and their surrounding ECs in the tumor niche occurred by interaction between Notch ligands (which included JAG1, DLL1, and DLL4 expressed by tumor ECs) and the Notch receptors, including Notch1 and Notch2. The latter were expressed by CSCs and the interaction increased self-renewal of CSCs and promoted tumor growth [200] (Figure 26.3). Inhibition of Notch signaling from the tumor vascular niche resulted in depletion of CSCs and inhibition of tumor growth. The existence of a tumor vascular niche has also been shown for head and neck cancer stem-like cells with approximately 80% of the CSCs located in close proximity (within 100 μm radius) of blood vessels in human squamous cell carcinoma (SCC) [201].

It seems that a contact-dependent juxtacrine interaction of neural CSCs with their niche is required to preserve the self-renewal potential of tumor stem cells. Assessment of interactions between primary brain tumor microvascular ECs (tMVECs) and glioma-propagating cells showed that only direct contact between the two cell types stimulated proliferation and expansion of the glioma cells as opposed to paracrine interactions between tMVECs and glioma cells when they were co-cultured in transwell chambers or ECM secreted by tMVECs. Only tMVECs

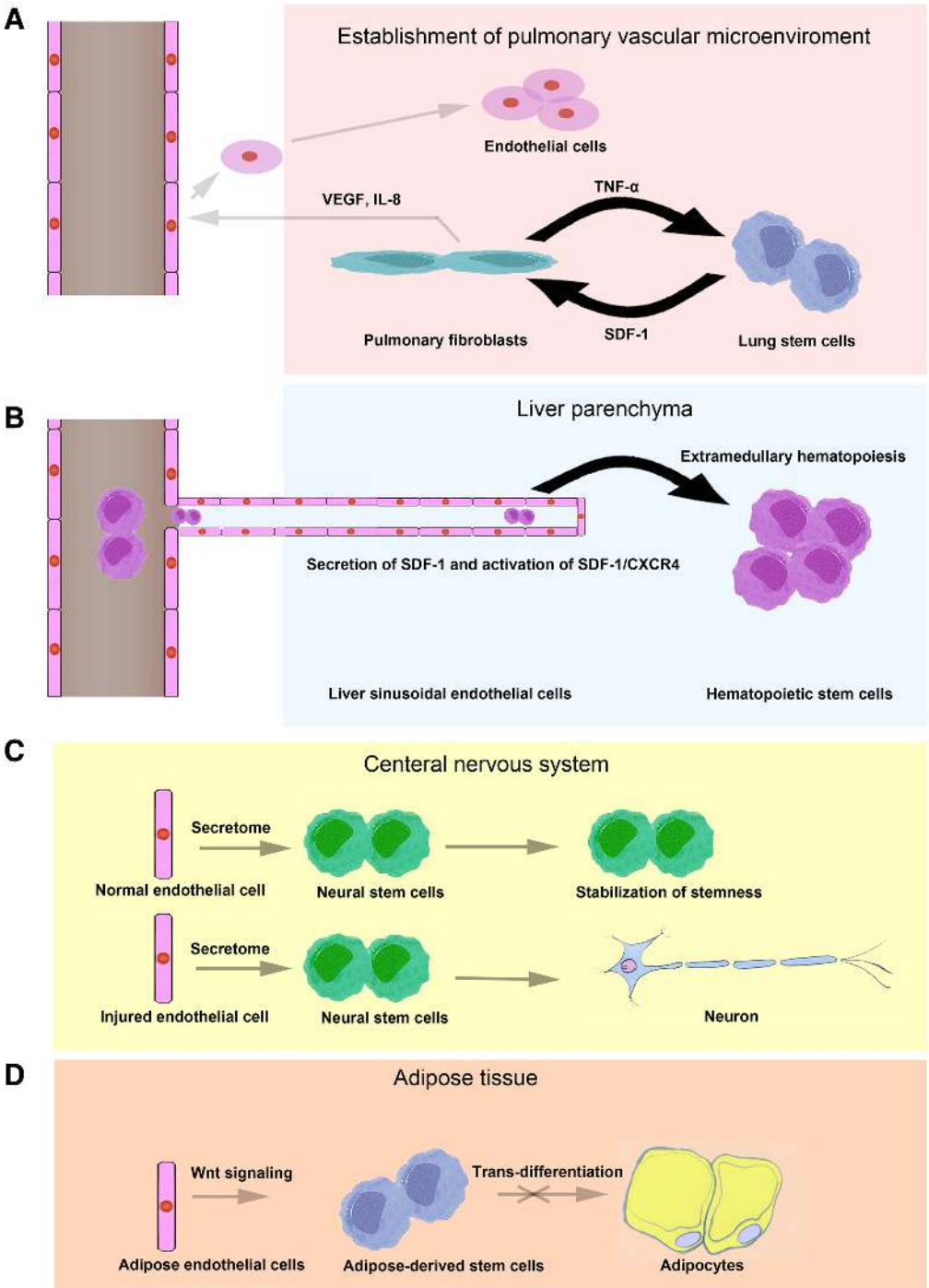



**Figure 26.5** Bidirectional EC–stem cell crosstalk leads to the development of niches with biological activity in different tissues under physiologic and pathologic conditions. (A) In the lung, development of a vascular niche by LSCs and fibroblasts directs stem cell growth and differentiation. (B) EC–progenitor cell interaction through SDF-1/CXCR4 controls physiologic and pathologic extramedullary hematopoiesis in the liver. (C) In the brain, a vascular niche exhibits different interactions with neural stem cells during physiologic and pathologic conditions. (D) In adipose tissue, a vascular niche maintains stem cell quiescence by Wnt signaling. (See color plate section.)

supported the expansion of glioma-propagating cells isolated from primary tumor specimens. HUVECs did not have a significant effect on the proliferation of the tumor cells [202]. Again, the SDF-1 (CXCL12)–CXCR4 signaling pathway was reported to direct the localization of glioma tumor cells in the vascular niche and mediate EC trophic function in glioblastoma multiforme, while depletion of CXCR4 in the glioma cell line U87 blocked its growth in xenografts transplanted intracranially [203].

It appears that there is bidirectional interplay between ECs and their surrounding cells in the vascular niche. Evidence shows that bidirectional interaction of tumor stem cells and ECs is an intricate microstructure that, on the one hand, supports the proliferation and expansion of neoplastic cells and, on the other hand, protects ECs. Brain tumor stem cells, which are supported in a protective vascular niche, stimulate angiogenesis through production of proangiogenic growth factors or directly contribute to tumor microvasculature through plasticity toward an endothelial lineage [204]. In gliomas, the tumor stem cells induced the synthesis of VEGF, which was associated with the development of aggressive neoplastic subpopulations in advanced stages of disease [205]. Moreover, crosstalk between ECs and their surrounding cells in the vascular niche is discernible in other situations. It has been demonstrated in injury to the white matter of the brain that secretion of MMP-9 by oligodendrocytes following upregulation of the proinflammatory cytokine IL-1β accelerated the angiogenic response in the oligovascular niche, which may have contributed to recovery of the white matter after brain injury [206]. There was coupled angiogenesis/neurogenesis crosstalk between cerebral ECs and subventricular zone NPCs after stroke injury, which was likely mediated by VEGF since blockage of VEGFR2 diminished the effect of the ischemia-activated ECs on NPC neurogenesis. Additionally, NPC-conditioned medium had an effect on endothelial angiogenesis [207]. Interactions between tumor cells and ECs in the vascular niche may influence other elements of the tumor. Crosstalk between tumor cells and ECs in medulloblastoma resulted in the secretion of macrophage migration inhibitory factor by tumor cells, which induced the tumor ECs to secrete the potent T lymphocyte chemoattractant RANTES, thus creating a chemotactic gradient guiding RANTES-receptor-expressing T cells to the tumor [208]. (Medulloblastoma is the most common malignant primary brain tumor in children, originating in the cerebellum or posterior fossa.)

MSCs have been shown to be capable of protecting the perivascular niche through stabilizing the vascular basement membrane [209]. MSCs undertake a supportive role for ECs in the vascular niche similar to that of pericytes [68,210,211]. MSCs secrete high levels of TIMP-1 and TIMP-2, while ECs mainly secrete TIMP-2 [209] (Figure 26.3). TIMPs are considered key protectors against degradation by MMP-2 and MMP-9 of the main matrix components of the vascular basement membrane, including laminins and collagen type IV [212,213]. Unlike ECs, MSCs increased TIMP-1 -expression when stressed by the proinflammatory cytokines IL-1β and TNF-α or by hypoxia. Secreted TIMP-1 in addition to TIMP-2 efficiently bound and inhibited MMP-2 under pathological conditions, highlighting the fact that MSCs as uniquely able to augment protection of the perivascular niche with secretion of multiple TIMPs [209].

Microparticles secreted by mesenchymal cell lines, but not microparticles from epithelial cell lines, contained high levels of angiogenic molecules, including PDGF, IL-8, and angiogenin, with the capability of endothelial activation and development of a vascular niche that promoted cancer cell proliferation, invasiveness, stem cell phenotype, and chemoresistance [214] (Figure 26.3). Moreover, induction of angiogenesis and promotion of a premetastatic niche was found during release of microvesicles by a $CD105^+$ MSC-like subset of human renal CSCs [215]. Microvesicles derived from $CD105^+$ CSCs modified the lung microenvironment after intravenous administration into SCID mice by enhancing the expression of VEGFR1, VEGF, MMP9, and MMP2 in lung tissue or in lung ECs, leading to the formation of a premetastatic niche [215–217]. Several microRNAs associated with tumor invasion and metastasis, including miR-29a, miR-650, and miR-151, were found to be shuttled by the $CD105^+$ microvesicles. A variety of mechanisms, including ligand–receptor interaction [134,218], direct cell–cell contact by gap junctions [219], or cell-to-cell communication through tunneling nanotubes [220,221] and exosome-mediated cell communication [222] have all been shown to be involved in cell–cell crosstalk in the tumor niche.

## 26.7 Determination of stem cell fate through crosstalk with endothelial cells

The fate of stem/stromal cells can be determined through bilateral interaction with ECs, which seems to be context dependent. In the central nervous system, actively proliferating type B and type C subventricular zone neural stem cells were found in close proximity to the vascular niche in the subventricular zone of the brain [223,224]. Activated type B and type C stem cells expressed high levels of EGFR and $\alpha_6$ integrin, which was mediated by SDF-1 secreted in the niche, resulting in migration of quiescent type B stem cells from the ependymal surface toward the blood vessel surface along with activation and proliferation of these cells and the generation of type C cells [196]. (Quiescent neural stem cells are type B. They are able to remain in the quiescent state due to the specific niches composed of blood vessels, astrocytes, microglia, ependymal cells, and ECM present within the brain. These niches provide nourishment, structural support, and protection for the stem cells until they are activated by external stimuli. Once activated, type B cells develop into type C cells, which are active proliferating intermediate cells, which then divide into neuroblasts consisting of type A cells.)

Co-culture of the human embryonic stem cell lines H1 and H9, as well as human induced pluripotent stem cells, with the transformed brain EC line bEnd.3 retained their pluripotency [225]. bEnd.3 and its conditioned medium, which is similar to that of primary mouse embryonic fibroblasts, expanded H9 ESCs with correct morphology and maintenance of expression of pluripotency markers. This makes bEnd.3 an appropriate feeder layer for prolonged culture of human pluripotent cells. In glioblastoma multiforme, brain ECs and their conditioned media protected the stemness of CSCs and the neurosphere-forming

ability of the tumor stem cells was sustained in the presence of the EC secretome but not with epithelial cell conditioned media [226]. These authors demonstrated a role for the mammalian target of rapamycin pathway as the means of active communication between ECs and CSCs and showed that the EC secretome supported the maintenance of glioblastoma stem-like cells. A combination of an anti-angiogenic agent (anti-VEGFR2) targeting the tumor vascular niche and chemotherapy was used in nude mice bearing subcutaneous xenografts of the C6 rat glioma cell line. A significant reduction in the fraction of tumor sphere-forming units was attained in the tumor, indicating that selective eradication of tumor stem cells can be achieved by targeting the tumor vascular niche, attesting to the ability of anti-angiogenic therapy to augment the efficacy of chemotherapy [227]. Similar results were obtained using the combination of the vascular disrupting agent combretastatin A1 diphosphate (OXi4503 or CA1P) with low-dose metronomic cyclophosphamide chemotherapy in xenografts of human breast cancer and melanoma that developed after injection of the 231/LM2-4 breast cancer cell line and MeWo melanoma cell line respectively [228]. Significant decreases in microvessel density, perfusion, and proliferation, accompanied by significant increases in tumor cell necrosis and apoptosis, were achieved by this protocol. (Metronomic chemotherapy refers mainly to the schedule of the chemotherapy, which consists of chronic, equally spaced, and (generally) low doses of various chemotherapeutic drugs without extended rest periods. This is markedly different from the current chemotherapy approach, which normally uses maximally tolerated doses of chemotherapeutic agents usually given as a number of courses over a period of months.)

Other studies, however, have reported that crosstalk between ECs and MSCs may prompt MSCs to exit the niche through induction of differentiation. Saleh *et al.* reported that paracrine factors secreted by HUVECs stimulated the proliferation of human MSCs and significantly enhanced their osteogenic differentiation [76]. Bioinformatic integration of gene expression datasets from HUVECs and MSCs suggested that a number of signaling pathways, including FGF, Wnt, BMP, PDGF, TGFβ, Notch, and Ephrin, might be involved in MSC–EC crosstalk [76]. A similar effect on the proliferation and osteogenic differentiation of MSCs by HUVECs has also been reported by others [87,229,230]. When different ratios of human MSCs and HUVECs were used in a co-culture system, a significant increase in the proliferation and osteogenic differentiation of MSCs was observed: augmentation in MSC proliferation occurred independently of relative cell ratios, whereas osteogenic induction, as assessed by alkaline phosphatase activity, was dependent on the cell : cell ratio. The highest alkaline phosphatase activity occurred in the 3 : 1 ratio of MSCs : HUVECs under nonosteogenic conditions [87]. Significantly enhanced MSC expansion with induction of osteogenesis using different ratios of MSCs to HUVECs was shown in another study with the highest proliferation rate of MSCs at a 1 : 4 MSC : HUVEC co-culture ratio [230].

VEGF appears important in osteoblastic differentiation of MSCs co-cultured with ECs, since inhibition of its receptors reduced the co-culture-induced osteoblastic activity [231]. Genetic modification of MSCs to overexpress VEGF resulted in inhibition of BMP2 expression in MSCs and inhibited osteogenesis *in vitro* and ectopic bone formation *in vivo*, highlighting VEGF as a potential inhibitor of BMP2 expression in MSCs' capability of altering the osteogenesis of MSCs [170]. Scrutiny of the role of VEGF in MSC differentiation showed that there is an intracrine mechanism by which VEGF regulates osteoblastic differentiation of MSCs [232]. Marrow MSCs from mice with conditional VEGF deficiency in osteoblastic precursor cells, or MSCs in which VEGF had been knocked down *in vitro*, exhibited a significant reduction in osteoblast differentiation and increased adipocyte differentiation that was independent of exogenous VEGF. Furthermore, there was a reciprocal interaction between VEGF and the nuclear envelope proteins lamin A/C in bone-marrow-derived MSCs in which lamin A stimulated VEGF protein expression while VEGF repressed levels of lamin A. The authors concluded that VEGF may induce osteoblastic differentiation of MSCs by stimulating the transcription factor RUNX2 and may control adipogenesis by suppressing the expression of peroxisome proliferator-activated receptor-γ2 (PPAR-γ2) [232]. In an attempt to further investigate the crosstalk between ECs and bone cells, noncontact co-culture of human osteoblast-like cells with HUVECs showed that osteoblasts released VEGF in response to prostaglandins, and ECs subsequently responded to VEGF with phosphorylation of VEGFR2, activation of ERK1/2, cyclooxygenase-2 induction, and release of prostanoids such as prostacyclin. These prostanoids may be key mediators in facilitating osteoblast–EC communication by enhancing the release of VEGF from osteoblasts [233]. However, interactions between these cells also appeared to depend on other factors, such as juxtacrine interactions or the distance of cells from each other. An osteoblast–EC contact co-culture model showed that the loss of VEGF sensitivity as well as abrogation of VEGF-mediated osteoblastic differentiation might be mediated by the action of prostaglandin $E_2$ with the possibility of restricting VEGF signaling to those ECs that were already in close proximity to osteoblasts [234].

Co-culture of human MSCs with HUVECs in a 3D spheroid system inhibited the proliferation and adipogenic differentiation of MSCs while selectively promoting osteogenic differentiation of MSCs. This indicated that, in an *in vivo* simulated 3D system, ECs maintained quiescence and facilitated niche exit via osteogenic differentiation [79]. Activation of endogenous Wnt and BMP signaling was demonstrated in MSCs by increased levels of immunoreactivity against downstream mediators of these signaling pathways. However, direct interactions with distinct differentiated cells may be a critical determinant of MSC fate. Although co-culture of MSCs with both ECs and dermal fibroblasts resulted in the appearance of α-SMA filaments in MSCs, these cells showed a pericyte-like morphology when co-cultured with ECs. A spindle-shaped myofibroblast-like morphology was

observed after co-culture with fibroblasts [78]. Direct cell–cell contact between MSCs and adult cardiomyocytes and smooth muscle cells specifically directed the fate of MSCs toward cardiomyocyte-like and smooth-muscle-like cells [235,236].

Endothelial interaction of human adipose-derived MSCs in the perivascular niche was found to maintain the stemness phenotype and inhibited the adipogenic differentiation of stem cells [237] (Figure 26.5D). Purified human perivascular stem cells from adipose tissue were shown to possess properties of MSCs [238,239]. Juxtacrine and paracrine interactions of ECs and adipose-derived MSCs resulted in modulation of stem cell fate through canonical Wnt signaling, predominantly by Wnt1, highlighting the relationship between MSCs and ECs in the regulation of adipogenesis in adipose tissue [237] (Figure 26.5D). Moreover, in porcine adipose-derived MSCs, inhibition of adipogenic differentiation was demonstrated by the activation of Wnt signaling via Wnt3a, which was shown to repress the mutual activation of CCAAT/enhancer-binding protein-α and PPAR-γ [240].

EC–stem-cell crosstalk may determine the fate of CSCs during tumor expansion. It was shown that interaction between ECs and CSCs from SCCs of the head and neck protected CSCs against anoikis and induced proliferation of these cells by VEGF secretion from ECs. (Anoikis is a form of programmed cell death that is induced by anchorage-dependent cells detaching from the surrounding extracellular matrix.) These findings indicated that the survival and self-renewal of head and neck SCCs occurs via EC-derived signals [241]. EC-induced phosphorylation of signal transducer and activator of transcription 3, Akt, and ERK in SCCs of the head and neck was mediated through upregulation of IL-6, IL-8, and EGF in ECs co-cultured with head and neck SCC cells, leading to increase in migration and inhibition of anoikis of the tumor cells [242].

## 26.8 Beneficial effects of mesenchymal stromal cell–endothelial cell interactions in some tissue pathologies

Long-term repeat positron emission tomography/computed tomography monitoring with the novel imaging agent 2′-deoxy-2′-[$^{18}$F]-fluoro-5-ethyl-1-β-D-arabinofuranosyluracil of MSCs retrovirally transduced with the sr39HSV1-tk gene after their intramyocardial transplantation in pigs showed that the peak of cell proliferation occurred at 33–35 days after injection in peri-infarct regions and in the major cardiac lymphatic vessels and lymph nodes [243]. Histochemical staining showed sr39HSV1-tk-expressing fibromyoblasts, lymphovascular cells, and microvascular and arterial endothelium, indicative of integration of transplanted MSCs into both lymphatic and vascular endothelium [243]. In a sex-mismatched cell transplantation model in miniswine, Y-chromosome-positive transplanted cells exhibited vascular differentiation of both vascular smooth muscle cells and ECs, contributing to large and small vessel formation in recipient female hearts [244]. Improved regional contractility and myocardial blood flow resulted in reduction of infarct size and improvement of cardiac function. Intramyocardial transplantation of MSCs dynamically changed the milieu of the injured myocardium toward a proangiogenic microenvironment by activation of VEGF signaling in the infarction and an increase in the Ang-2 : Ang-1 ratio, which resulted in reduction of phosphorylated Tie-2 [2].

In a unilateral ureteric obstruction model in mice, accumulation of a variety of stem/progenitor cells, including MSCs, HSCs, and EPCs, attenuated the deleterious tubulointerstitial scarring and contributed to regeneration of the renal parenchyma after release of the ureteric obstruction. This was thought due to an EC–stem-cell interaction likely through the SDF-1/CXCR-4 axis [245]. In acute kidney injury, paracrine and microvesicle-mediated production of proangiogenic factors, including VEGF, IGF, FGF-2, as well as erythropoietin, has been proposed as an important mechanism in MSC-related renoprotection [246].

MSC–EC interactions, particularly those mediated by paracrine mechanisms, significantly enhanced recruitment of both VE-cadherin and β-catenin to the plasma membrane of ECs. This established a VE-cadherin–β-catenin interaction with eventual establishment of functional adherence junctions in these cells. This in turn led to a reduction in endothelial permeability [81] (Figure 26.2). This interaction between MSCs and ECs also reduced blood–brain barrier permeability in mice with traumatic brain injury. In a murine model of stroke injury, expression of Ang-1, Tie-2, VEGF/VEGFR2 and Occludin was induced by MSCs and led to promotion of angiogenesis and establishment of vascular integrity [247]. In this context, Ang-1/Tie-2 signaling was found to be capable of protecting MSCs and ECs against adverse conditions such as serum deprivation and hypoxia-induced apoptosis by Akt phosphorylation, decreased activation of caspase-9 and -3, and an increased Bcl-2 : Bax ratio [248].

## References

1 Urbán, V.S., Kiss, J., Kovács, J., *et al.* Mesenchymal stromal cells cooperate with bone marrow cells in therapy of diabetes. *Stem Cells* 2008;**26**(1):244–253.

2 Rahbarghazi, R., Nassiri, S.M., Ahmadi, S.H., *et al.* Dynamic induction of pro-angiogenic milieu after transplantation of marrow-derived mesenchymal stromal cells in experimental myocardial infarction. *Int J Cardiol* 2014;**173**(3):453–466.

3 Ley, K., Laudanna, C., Cybulsky, M.I., Nourshargh, S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;**7**(9):678–689.

4 Teo, G.S., Ankrum, J.A., Martinelli, R., *et al.* Mesenchymal stromal cells transmigrate between and directly through tumor necrosis factor-α-activated endothelial cells via both leukocyte-like and novel mechanisms. *Stem Cells* 2012;**30**(11):2472–2486.

5 Beyth, S., Z.Borovsky, Mevorach, D., *et al*. Human mesenchymal stromal cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;**105**(5):2214–2219.

6 Kasper, G., Dankert, N., Tuischer, J., *et al*. Mesenchymal stromal cells regulate angiogenesis according to their mechanical environment. *Stem Cells* 2007;**25**(4):903–910.

7 Semon, J., Nagy, L., Llamas, C.B., *et al*. Integrin expression and integrin-mediated adhesion *in vitro* of human multipotent stromal cells (MSCs) to endothelial cells from various blood vessels. *Cell Tissue Res* 2010;**341**(1):147–158.

8 Schneider, D., Engelman, D.M. Involvement of transmembrane domain interactions in signal transduction by α/β integrins. *J Biol Chem* 2004;**279**(11):9840–9846.

9 Watt, F.M. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J* 2002;**21**(15):3919–3926.

10 Docheva, D., Popov, C., Mutschler, W., Schieker, M. Human mesenchymal stromal cells in contact with their environment: surface characteristics and the integrin system. *J Cell Mol Med* 2007;**11**(1):21–38.

11 Ip, J.E., Wu, Y., Huang, J., *et al*. Mesenchymal stromal cells use integrin $\beta_1$ not CXC chemokine receptor 4 for myocardial migration and engraftment. *Mol Biol Cell* 2007;**18**(8): 2873–2882.

12 Zou, C., Song, G., Luo, Q., *et al*. Mesenchymal stromal cells require integrin $\beta_1$ for directed migration induced by osteopontin *in vitro*. *In Vitro Cell Dev Biol Anim* 2011;**47**(3):241–250.

13 Veevers-Lowe, J., Ball, S.G., Shuttleworth, A., Kielty, C.M. Mesenchymal stem cell migration is regulated by fibronectin through $\alpha_5\beta_1$-integrin-mediated activation of PDGFR-β and potentiation of growth factor signals. *J Cell Sci* 2011;**124**(8):1288–1300.

14 Tabe, Y., L.Jin, L., Tsutsumi-Ishii, Y., *et al*. Activation of integrin-linked kinase is a critical prosurvival pathway induced in leukemic cells by bone marrow-derived stromal cells. *Cancer Res* 2007;**67** (2):684–694.

15 Artavanis-Tsakonas, S., Rand, M.D., Lake, R.J. Notch signaling: cell fate control and signal integration in development. *Science* 1999;**284**(5415):770–776.

16 Docheva, D., Haasters, F., Schieker, M. Mesenchymal stromal cells and their cell surface receptors. *Curr Rheumatol Rev* 2008;**4**(3): 155–160.

17 Li, Y., Hiroi, Y., Liao, J.K. Notch signaling as an important mediator of cardiac repair and regeneration after myocardial infarction. *Trends Cardiovasc Med* 2010;**20**(7):228–231.

18 Xie, J., Wang, W., Si, J.W., *et al*. Notch signaling regulates CXCR4 expression and the migration of mesenchymal stromal cells. *Cell Immunol* 2013;**281**(1):68–75.

19 Li, Y., Hiroi, Y., Ngoy, S., *et al*. Notch1 in bone marrow-derived cells mediates cardiac repair after myocardial infarction. *Circulation* 2011;**123**(8):866–876.

20 Isobe, M., Yagita, H., Okumura, K., Ihara, A. Specific acceptance of cardiac allograft after treatment with antibodies to ICAM-1 and LFA-1. *Science* 1992;**255**(5048):1125–1127.

21 Segers, V.F., Van Riet, I., Andries, L.J., *et al*. Mesenchymal stem cell adhesion to cardiac microvascular endothelium: activators and mechanisms. *Am J Physiol Heart Circ Physiol* 2006;**290**(4): H1370–H1377.

22 Croitoru-Lamoury, J., Lamoury, F.M., Zaunders, J.J., *et al*. Human mesenchymal stromal cells constitutively express chemokines and chemokine receptors that can be upregulated by cytokines, IFN-β, and copaxone. *J Interferon Cytokine Res* 2007;**27**(1):53–64.

23 Duijvestein, M., Wildenberg, M.E., Welling, M.M., *et al*. Pretreatment with interferon-γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells* 2011;**29**(10):1549–1558.

24 Zhao, W., Loh, W., Droujinine, I.A., *et al*. Mimicking the inflammatory cell adhesion cascade by nucleic acid aptamer programmed cell–cell interactions. *FASEB J* 2011;**25**(9):3045–3056.

25 Rüster, B., Göttig, S., Ludwig, R.J., *et al*. Mesenchymal stromal cells display coordinated rolling and adhesion behavior on endothelial cells. *Blood* 2006;**108**(12):3938–3944.

26 Chamberlain, G., Smit, H., Rainger, G.E., Middleton, J. Mesenchymal stromal cells exhibit firm adhesion, crawling, spreading and transmigration across aortic endothelial cells: effects of chemokines and shear. *PloS ONE* 2011;**6**(9):e25663.

27 Phillips, J.A., Mortensen, L.J., Ruiz, J.P., *et al*. Advances in single-cell tracking of mesenchymal stromal cells (MSCs) during musculoskeletal regeneration. *Orthop J Harv Med Sch* 2012;**14**:22–28.

28 Zhu, H., Mitsuhashi, N., Klein, A., *et al*. The role of the hyaluronan receptor CD44 in mesenchymal stem cell migration in the extracellular matrix. *Stem Cells* 2006;**24**(4):928–935.

29 Sackstein, R., Merzaban, J.S., Cain, D.W., *et al*. *Ex vivo* glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nat Med* 2008;**14**(2):181–187.

30 Potapova, I.A., Cohen, I.S., Doronin, S.V. Von Willebrand factor increases endothelial cell adhesiveness for human mesenchymal stromal cells by activating p38 mitogen-activated protein kinase. *Stem Cell Res Ther* 2010;**1**(5):35–40.

31 Malinowski, M., Pietraszek, K., Perreau, C., *et al*. Effect of lumican on the migration of human mesenchymal stromal cells and endothelial progenitor cells: involvement of matrix metalloproteinase-14. *PLoS ONE* 2012;**7**(12):e50709.

32 Otsu, K., Das, S., Houser, S.D., *et al*. Concentration-dependent inhibition of angiogenesis by mesenchymal stromal cells. *Blood* 2009;**113**(18):4197–4205.

33 Haider, H.Kh., Jiang, S., Idris, N.M., Ashraf, M. IGF-1-overexpressing mesenchymal stromal cells accelerate bone marrow stem cell mobilization via paracrine activation of SDF-1α/CXCR4 signaling to promote myocardial repair. *Circ Res* 2008;**103**(11):1300–1308.

34 Reymond, N., d'Água, B.B., Ridley, A.J. Crossing the endothelial barrier during metastasis. *Nat Rev Cancer* 2013;**13**(12):858–870.

35 Salem, H.K., Thiemermann, C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* 2010;**28**(3):585–596.

36 Zhang, F., Wang, C., Wang, H., *et al*. Ox-LDL promotes migration and adhesion of bone marrow-derived mesenchymal stromal cells via regulation of MCP-1 expression. *Mediators Inflamm* 2013;**2013**: 691023.

37 Sheibani, N., Frazier, W. Thrombospondin-1, PECAM-1, and regulation of angiogenesis. *Histol Histopathol* 1999;**14**(1):285–294.

38 Sheibani, N., Sorenson, C.M., Frazier, W.A. Differential modulation of cadherin-mediated cell–cell adhesion by platelet endothelial cell adhesion molecule-1 isoforms through activation of extracellular regulated kinases. *Mol Biol Cell* 2000;**11**(8):2793–2802.

39 Wright, T., Leach, L., Shaw, P.E., Jones, P. Dynamics of vascular endothelial-cadherin and β-catenin localization by vascular endothelial growth factor-induced angiogenesis in human umbilical vein cells. *Exp Cell Res* 2002;**280**(2):159–168.

40 Linder, S. Invadosomes at a glance. *J Cell Sci* 2009;**122**(17): 3009–3013.

41 Schmidt, A., Ladage, D., Steingen, C., *et al*. Mesenchymal stromal cells transmigrate over the endothelial barrier. *Eur J Cell Biol* 2006;**85**(11):1179–1188.

42 Allen, S.J., Crown, S.E., Handel, T.M. Chemokine: receptor structure, interactions, and antagonism. *Annu Rev Immunol* 2007;**25**: 787–820.

43 Koltsova, E., Ley, K. Tyrosine sulfation of leukocyte adhesion molecules and chemokine receptors promotes atherosclerosis. *Arterioscler Thromb Vasc Biol* 2009;**29**(11):1709–1711.

44 Ji, J.F., He, B.P., Dheen, S.T., Tay, S.S. Interactions of chemokines and chemokine receptors mediate the migration of mesenchymal stromal cells to the impaired site in the brain after hypoglossal nerve injury. *Stem Cells* 2004;**22**(3):415–427.

45 Ringe, J., Strassburg, S., Neumann, K, *et al*. Towards *in situ* tissue repair: human mesenchymal stromal cells express chemokine receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 2007;**101**(1): 135–146.

46 Smith, H., Whittall, C., Weksler, B., Middleton, J. Chemokines stimulate bidirectional migration of human mesenchymal stromal cells across bone marrow endothelial cells. *Stem Cells Dev* 2011;**21** (3):476–486.

47 Marastoni, S., Ligresti, G., Lorenzon, E., *et al*. Extracellular matrix: a matter of life and death. *Connect Tissue Res* 2008;**49**(3–4):203–206.

48 Choi, B.-H., Choi, Y.S., Kang, D.G., *et al*. Cell behavior on extracellular matrix mimic materials based on mussel adhesive protein fused with functional peptides. *Biomaterials* 2010;**31**(34): 8980–8988.

49 Ode, A., Duda, G.N., Glaeser, J.D., *et al*. Toward biomimetic materials in bone regeneration: Functional behavior of mesenchymal stromal cells on a broad spectrum of extracellular matrix components. *J Biomed Mater Res Part A* 2010;**95**(4):1114–1124.

50 Xiao, G., Gopalakrishnan, R., Jiang, D., *et al*. Bone morphogenetic proteins, extracellular matrix, and mitogen-activated protein kinase signaling pathways are required for osteoblast-specific gene expression and differentiation in MC3T3-E1 cells. *J Bone Miner Res* 2002;**17**(1):101–110.

51 Wu, S., Wells, A., Griffith, L.G., Lauffenburger, D.A. Controlling multipotent stromal cell migration by integrating "course-graining" materials and "fine-tuning" small molecules via decision tree signal–response modeling. *Biomaterials* 2011;**32**(30):7524–7531.

52 Zhao, Z., Watt, C., Karystinou, A., *et al*. Directed migration of human bone marrow mesenchymal stromal cells in a physiological direct current electric field. *Eur Cell Mater* 2011;**22**:344–358.

53 Johansson, U., Rasmusson, I., Niclou, S.P., *et al*. Formation of composite endothelial cell–mesenchymal stem cell islets a novel approach to promote islet revascularization. *Diabetes* 2008;**57**(9): 2393–2401.

54 Li, N., Lu, X., Zhao, X., *et al*. Endothelial nitric oxide synthase promotes bone marrow stromal cell migration to the ischemic myocardium via upregulation of stromal cell-derived factor-1α. *Stem Cells* 2009;**27**(4):961–970.

55 Aguirre, A., Planell, J., Engel, E. Dynamics of bone marrow-derived endothelial progenitor cell/mesenchymal stem cell interaction in co-culture and its implications in angiogenesis. *Biochem Biophys Res Commun* 2010;**400**(2):284–291.

56 Nassiri, S.M., Rahbarghazi, R. Interactions of mesenchymal stromal cells with endothelial cells. *Stem Cells Dev* 2014;**23**(4): 319–332.

57 Rahbarghazi, R., Nassiri, S.M., Khazraiinia, P, *et al*. Juxtacrine and paracrine interactions of rat marrow-derived mesenchymal stromal cells, muscle-derived satellite cells, and neonatal cardiomyocytes with endothelial cells in angiogenesis dynamics. *Stem Cells Dev* 2012;**22**(6):855–865.

58 Thin Luu, N., McGettrick, H.M., Buckley, C.D., *et al*. Crosstalk between mesenchymal stromal cells and endothelial cells leads to downregulation of cytokine-induced leukocyte recruitment. *Stem Cells* 2013;**31**(12):2690–2702.

59 Wang, N., Zhang, R., Wang, S.J., *et al*. Vascular endothelial growth factor stimulates endothelial differentiation from mesenchymal stromal cells via Rho/myocardin-related transcription factor-A signaling pathway. *Int J Biochem Cell Biol* 2013;**45**(7):1447–1456.

60 Hall, A., Nobes, C.D. Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Phil Trans R Soc London Ser B Biol Sci* 2000;**355**(1399):965–970.

61 Zhao, Z.-S., Manser, E. PAK and other Rho-associated kinases – effectors with surprisingly diverse mechanisms of regulation. *Biochem J* 2005;**386**:201–214.

62 Bryan, B.A., Dennstedt, E., Mitchell, D.C., *et al*. RhoA/ROCK signaling is essential for multiple aspects of VEGF-mediated angiogenesis. *FASEB J* 2010;**24**(9):3186–3195.

63 Calzado-Martín, A., Méndez-Vilas, A., Multigner, M., *et al*. On the role of RhoA/ROCK signaling in contact guidance of bone-forming cells on anisotropic Ti6Al4V surfaces. *Acta Biomater* 2011; **7**(4):1890–1901.

64 Ren, X.D., Kiosses, W.B., Schwartz, M.A. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J* 1999;**18**(3):578–585.

65 Lin, M.-N., Shang, D.-S., Sun, W., *et al*. Involvement of PI3K and ROCK signaling pathways in migration of bone marrow-derived mesenchymal stromal cells through human brain microvascular endothelial cell monolayers. *Brain Res* 2013;**1513**:1–8.

66 Da Silva Meirelles, L., Caplan, A.I., Nardi, N.B. In search of the *in vivo* identity of mesenchymal stromal cells. *Stem Cells* 2008;**26** (9):2287–2299.

67 Boyd, N.L., Nunes, S.S., Jokinen, J.D., *et al*. Microvascular mural cell functionality of human embryonic stem cell-derived mesenchymal cells. *Tissue Eng Part A* 2011;**17**(11–12):1537–1548.

68 Crisan, M., Yap, S., Casteilla, L., *et al*. A perivascular origin for mesenchymal stromal cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

69 Paul, J.D., Coulombe, K.L., Toth, P.T., *et al*. SLIT3–ROBO4 activation promotes vascular network formation in human engineered tissue and angiogenesis *in vivo*. *J Mol Cell Cardiol* 2013;**64**:124–131.

70 Marlow, R., Binnewies, M., Sorensen, L.K., *et al*. Vascular Robo4 restricts proangiogenic VEGF signaling in breast. *Proc Natl Acad Sci U S A* 2010;**107**(23):10520–10525.

71 Pedersen, T.O., Blois, A.L., Xue, Y., *et al*. Mesenchymal stromal cells induce endothelial cell quiescence and promote capillary formation. *Stem Cell Res Ther* 2014;**5**(1):1–10.

72 Blocki, A., Wang, Y., Koch, M., *et al*. Not all MSCs can act as pericytes: functional *in vitro* assays to distinguish pericytes from other mesenchymal stromal cells in angiogenesis. *Stem Cells Dev* 2013;**22**(17):2347–2355.

73 Schlosser, S., Dennler, C., Schweizer, R., *et al.* Paracrine effects of mesenchymal stromal cells enhance vascular regeneration in ischemic murine skin. *Microvasc Res* 2012;**83**(3):267–275.

74 Lin, R.-Z., Moreno-Luna, R., Zhou, B., *et al.* Equal modulation of endothelial cell function by four distinct tissue-specific mesenchymal stromal cells. *Angiogenesis* 2012;**15**(3):443–455.

75 Caplan, A.I. All MSCs are pericytes? *Cell Stem Cell* 2008;**3**(3): 229–230.

76 Saleh, F.A., Whyte, M., Ashton, P., Genever, P.G. Regulation of mesenchymal stem cell activity by endothelial cells. *Stem Cells Dev* 2010;**20**(3):391–403.

77 Kalinina, N., Sysoeva, V.Y., Rubina, K.A., *et al.* Mesenchymal stromal cells in tissue growth and repair. *Acta Naturae* 2011;**3**(4): 30–37.

78 Ball, S.G., Shuttleworth, A.C., Kielty, C.M. Direct cell contact influences bone marrow mesenchymal stem cell fate. *Int J Biochem Cell Biol* 2004;**36**(4):714–727.

79 Saleh, F., Whyte, M., Genever, P.G. Effects of endothelial cells on human mesenchymal stem cell activity in a three-dimensional *in vitro* model. *Eur Cell Mater* 2011;**22**:242–257.

80 Pati, S., Gerber, M.H., Menge, T.D., *et al.* Bone marrow derived mesenchymal stromal cells inhibit inflammation and preserve vascular endothelial integrity in the lungs after hemorrhagic shock. *PLoS ONE* 2011;**6**(9):e25171.

81 Pati, S., Khakoo, A.Y., Zhao, J., *et al.* Human mesenchymal stromal cells inhibit vascular permeability by modulating vascular endothelial cadherin/β-catenin signaling. *Stem Cells Dev* 2010;**20**(1): 89–101.

82 Menge, T., Gerber, M., Wataha, K., *et al.* Human mesenchymal stromal cells inhibit endothelial proliferation and angiogenesis via cell–cell contact through modulation of the VE-cadherin/β-catenin signaling pathway. *Stem Cells Dev* 2012;**22**(1):148–157.

83 Stenman, J.M., Rajagopal, J., Carroll, T.J., *et al.* Canonical Wnt signaling regulates organ-specific assembly and differentiation of CNS vasculature. *Science* 2008;**322**(5905):1247–1250.

84 Dufourcq, P., Descamps, B., Tojais, N.F., *et al.* Secreted frizzled-related protein-1 enhances mesenchymal stem cell function in angiogenesis and contributes to neovessel maturation. *Stem Cells* 2008;**26**(11):2991–3001.

85 Alfaro, M.P., Pagni, M., Vincent, A., *et al.* The Wnt modulator sFRP2 enhances mesenchymal stem cell engraftment, granulation tissue formation and myocardial repair. *Proc Natl Acad Sci U S A* 2008;**105**(47):18366–18371.

86 Duffy, G.P., D'Arcy S., Ashan, T., *et al.* Mesenchymal stromal cells overexpressing ephrin-B2 rapidly adopt an early endothelial phenotype with simultaneous reduction of osteogenic potential. *Tissue Eng Part A* 2010;**16**(9):2755–2768.

87 Bidarra, S.J., Barrias, C.C., Barbosa, M.A., *et al.* Phenotypic and proliferative modulation of human mesenchymal stromal cells via crosstalk with endothelial cells. *Stem Cell Res* 2011;**7**(3): 186–197.

88 Jiménez, E., Vicente, A., Sacedón, R., *et al.* Distinct mechanisms contribute to generate and change the CD4: CD8 cell ratio during thymus development: a role for the Notch ligand, *Jagged1*. *J Immunol* 2001;**166**(10):5898–5908.

89 Baraniak, P.R., McDevitt, T.C. Stem cell paracrine actions and tissue regeneration. *Regen Med* 2010;**5**(1):121–143.

90 El Andaloussi, S., Mäger, I., Breakefield, X.O., Wood, M.J. Extracellular vesicles: biology and emerging therapeutic opportunities. *Nat Rev Drug Discov* 2013;**12**(5):347–357.

91 György, B., Szabó, T.G., *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;**68**(16):2667–2688.

92 Zhang, H.-C., Liu, X.-B., Huang, S., *et al.* Microvesicles derived from human umbilical cord mesenchymal stromal cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev* 2012;**21**(18):3289–3297.

93 Li, T., Yan, Y., Wang, B., *et al.* Exosomes derived from human umbilical cord mesenchymal stromal cells alleviate liver fibrosis. *Stem Cells Dev* 2012;**22**(6):845–854.

94 Baglio, S.R., Pegtel, D.M., Baldini, N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012;**3**:359.

95 Choi, Y.-A., Lim, J., Kim, K.M., *et al.* Secretome analysis of human BMSCs and identification of SMOC1 as an important ECM protein in osteoblast differentiation. *J Proteome Res* 2010;**9**(6):2946–2956.

96 Kim, H.-S., Choi, D.-Y., Yun, S.J., *et al.* Proteomic analysis of microvesicles derived from human mesenchymal stromal cells. *J Proteome Res* 2011;**11**(2):839–849.

97 Salomon, C., Ryan, J., Sobrevia, L., *et al.* Exosomal signaling during hypoxia mediates microvascular endothelial cell migration and vasculogenesis. *PLoS ONE* 2013;**8**(7):e68451.

98 Lee, J.-K., Park, S.-R., Jung, P.K., *et al.* Exosomes derived from mesenchymal stromal cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* 2013;**8** (12):e84256.

99 Dignat-George, F., Boulanger, C.M. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* 2011;**31**(1):27–33.

100 Singh, N., Van Craeyveld, E., Tiwa, M., *et al.* Circulating apoptotic endothelial cells and apoptotic endothelial microparticles independently predict the presence of cardiac allograft vasculopathy. *J Am Coll Cardiol* 2012;**60**(4):324–331.

101 Chironi, G.N., Boulanger, C.M., Simon, A., *et al.* Endothelial microparticles in diseases. *Cell Tissue Res* 2009;**335**(1):143–151.

102 Leroyer, A.S., Ebrahimian, T.G., Cochain, C., *et al.* Microparticles from ischemic muscle promotes postnatal vasculogenesis. *Circulation* 2009;**119**(21):2808–2817.

103 Xin, H., Li, Y., Cui, Y., *et al.* Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013;**33**:1711–1715.

104 Xin, H., Li, Y., Liu, Z., *et al.* MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013;**31**(12):2737–2746.

105 Lai, R.C., Arslan, F., Lee, M.M., *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**(3):214–222.

106 Zhu, Y.G., Feng, X.M., Abbot, J., *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;**32**(1):116–125.

107 Maumus, M., Jorgensen, C., Noël, D. Mesenchymal stromal cells in regenerative medicine applied to rheumatic diseases: role of secretome and exosomes. *Biochimie* 2013;**95**(12):2229–2234.

108 Braccioli, L., vanVelthoven, C., Heijnen, C.J. Exosomes: a new weapon to treat the central nervous system. *Mol Neurobiol* 2014;**49**(1):113–119.

109 Raisi, A., Azizi, S., Delirezh, N., *et al*. The mesenchymal stem cell-derived microvesicles enhance sciatic nerve regeneration in rat: a novel approach in peripheral nerve cell therapy. *J Trauma Acute Care Surg* **201476**(4):991–997.

110 Katakowski, M., Buller, B., Zheng, X., *et al*. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett* 2013;**335**(1):201–204.

111 Roccaro, A.M., Sacco, A., Maiso, P., *et al*. BM mesenchymal stromal cell-derived exosomes facilitate multiple myeloma progression. *J Clin Invest* 2013;**123**(4):1542–1545.

112 Katsuda, T., Tsuchiya, R., Kosaka, N., *et al*. Human adipose tissue-derived mesenchymal stromal cells secrete functional neprilysin-bound exosomes. *Sci Rep* 2013;**3**:1197.

113 Kaigler, D., Krebsbach, P.H., Polverini, P.J., Mooney, D.J. Role of vascular endothelial growth factor in bone marrow stromal cell modulation of endothelial cells. *Tissue Eng* 2003;**9**(1):95–103.

114 Findley, C.M., Cudmore, M.J., Ahmed, A., Kontos, C.D. VEGF induces Tie2 shedding via a phosphoinositide 3-kinase/akt-dependent pathway to modulate Tie2 signaling. *Arterioscler Thromb Vasc Biol* 2007;**27**(12):2619–2626.

115 Leroux, L., Descamps, B., Tojais, N.F., *et al*. Hypoxia preconditioned mesenchymal stromal cells improve vascular and skeletal muscle fiber regeneration after ischemia through a Wnt4-dependent pathway. *Mol Ther* **201018**(8):1545–1552.

116 Chang, J., Sonoyama, W., Wang, Z., *et al*. Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stromal cells in craniofacial defects through activation of p38 MAPK. *J Biol Chem* 2007;**282**(42):30938–30948.

117 Karow, M., Popp, T., Egea, V., *et al*. Wnt signalling in mouse mesenchymal stromal cells: impact on proliferation, invasion and MMP expression. *J Cell Mol Med* 2009;**13**(8B):2506–2520.

118 Clause, K.C., Liu, L.J., Tobita, K. Directed stem cell differentiation: the role of physical forces. *Cell Commun Adhes* 2010;**17**(2):48–54.

119 Sadoshima, J., Izumo, S. Mechanical stretch rapidly activates multiple signal transduction pathways in cardiac myocytes: potential involvement of an autocrine/paracrine mechanism. *EMBO J* 1993;**12**(4):1681–1692.

120 Kasper, G., Glaeser, J.D., Geissler, S., *et al*. Matrix metalloprotease activity is an essential link between mechanical stimulus and mesenchymal stem cell behavior. *Stem Cells* 2007;**25**(8):1985–1994.

121 Ghajar, C.M., Blevins, K.S., Hughes, C.C., *et al*. Mesenchymal stromal cells enhance angiogenesis in mechanically viable prevascularized tissues via early matrix metalloproteinase upregulation. *Tissue Eng* 2006;**12**(10):2875–2888.

122 Liu, Y., Han, Z.P., Zhang, S.S., *et al*. Effects of inflammatory factors on mesenchymal stromal cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem* 2011;**286**(28):25007–25015.

123 Zhang, T., Lee, Y.W., Rui, Y.F., *et al*. Bone marrow-derived mesenchymal stromal cells promote growth and angiogenesis of breast and prostate tumors. *Stem Cell Res Ther* 2013;**4**(3):70.

124 Suzuki, K., Sun, R., Origuchi, M., *et al*. Mesenchymal stromal cells promote tumor growth through the enhancement of neovascularization. *Mol Med* 2011;**17**(7–8):579–587.

125 De Luca, A., Lamura, L., Gallo, M., *et al*. Mesenchymal stem cell-derived interleukin-6 and vascular endothelial growth factor promote breast cancer cell migration. *J Cell Biochem* 2012;**113**(11):3363–3370.

126 MartinF.T., Dwyer, R.M., Kelly, J., *et al*. Potential role of mesenchymal stromal cells (MSCs) in the breast tumour microenvironment: stimulation of epithelial to mesenchymal transition (EMT). *Breast Cancer Res Treat* 2010;**124**(2):317–326.

127 Comsa, S., Ciuculescu, F., Raica, M. Mesenchymal stem cell-tumor cell cooperation in breast cancer vasculogenesis. *Mol Med Rep* 2012;**5**(5):1175–1180.

128 Szebeni, G.J., Kriston-Pál, É., Blazsó, P., *et al*. Identification of galectin-1 as a critical factor in function of mouse mesenchymal stromal cell-mediated tumor promotion. *PLoS ONE* 2012;**7**(7):e41372.

129 Mathieu, V., deLassalle, E.M., Toelen, J., *et al*. Galectin-1 in melanoma biology and related neo-angiogenesis processes. *J Invest Dermatol* 2012;**132**(9):2245–2254.

130 Dhar, K., Dhar, G., Majumder, M., *et al*. Tumor cell-derived PDGF-B potentiates mouse mesenchymal stromal cells-pericytes transition and recruitment through an interaction with NRP-1. *Mol Cancer* 2010;**9**(5):209–221.

131 Kumar, A.H.S., Metharom, P., Schmeckpeper, J., *et al*. Bone marrow-derived CX3CR1 progenitors contribute to neointimal smooth muscle cells via fractalkine CX3CR1 interaction. *FASEB J* 2010;**24**(1):81–92.

132 Coffelt, S.B., Marini, F.C., Watson, K., *et al*. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc Natl Acad Sci U S A* **106**(10):3806–3811.

133 Chaturvedi, P., Gilkes, D.M., Wong, C.C., *et al*. Hypoxia-inducible factor-dependent breast cancer–mesenchymal stem cell bidirectional signaling promotes metastasis. *J Clin Invest* 2013;**123**(1):189–205.

134 Karnoub, A.E., Dash, A.B., Vo, A.P., *et al*. Mesenchymal stromal cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**(7162):557–563.

135 Bergfeld, S., DeClerck, Y. Bone marrow-derived mesenchymal stromal cells and the tumor microenvironment. *Cancer Metastasis Rev* 2010;**29**(2):249–261.

136 Abarrategi, A., Mariñas-Pardo, L., Mirones, I., *et al*. Mesenchymal niches of bone marrow in cancer. *Clin Transl Oncol* **13**(9):611–616.

137 Mi, Z., SBhattacharya, D., Kim, V.M., *et al*. Osteopontin promotes CCL5-mesenchymal stromal cell-mediated breast cancer metastasis. *Carcinogenesis* 2011;**2**(4):477–487.

138 Paunescu, V., Bojin, F.M., Tatu, C.A., *et al*. Tumour-associated fibroblasts and mesenchymal stromal cells: more similarities than differences. *J Cell Mol Med* 2011;**15**(3):635–646.

139 Jung, Y., Kim, J.K., Shiozawa, Y., *et al*. Recruitment of mesenchymal stromal cells into prostate tumours promotes metastasis. *Nat Commun* 2013;**4**:1795.

140 Kitadai, Y., Sasaki, T., Kuwai, T., *et al*. Targeting the expression of platelet-derived growth factor receptor by reactive stroma inhibits growth and metastasis of human colon carcinoma. *Am J Pathol* 2006;**169**(6):2054–2065.

141 Sumida, T., Kitadai, Y., Shinagawa, K., *et al*. Anti-stromal therapy with imatinib inhibits growth and metastasis of gastric carcinoma

in an orthotopic nude mouse model. *Int J Cancer* 2011;**128**(9): 2050–2062.

142 Shinagawa, K., Kitadai, Y., Tanaka, M., *et al*. Stroma-directed imatinib therapy impairs the tumor-promoting effect of bone marrow-derived mesenchymal stromal cells in an orthotopic transplantation model of colon cancer. *Int J Cancer* 2013;**132**(4): 813–823.

143 Akimoto, K., Kimura, K., Nagano, M., *et al*. Umbilical cord blood-derived mesenchymal stromal cells inhibit, but adipose tissue-derived mesenchymal stromal cells promote, glioblastoma multiforme proliferation. *Stem Cells Dev* 2012;**22**(9):1370–1386.

144 Kéramidas, M., deFraipont, F., Karageorgis, A., *et al*. The dual effect of mesenchymal stromal cells on tumour growth and tumour angiogenesis. *Stem Cell Res Ther* 2013;**4**:41.

145 Ho, I.A., Toh, H.C., Ng, W.H., *et al*. Human bone marrow-derived mesenchymal stromal cells suppress human glioma growth through inhibition of angiogenesis. *Stem Cells* 2013;**31**(1): 146–155.

146 Dasari, V.R., Kaur, K., Velpula, K.K., *et al*. Upregulation of PTEN in glioma cells by cord blood mesenchymal stromal cells inhibits migration via downregulation of the PI3K/Akt pathway. *PLoS ONE* 2010;**5**(4):e10350.

147 Oswald, J., Boxberger, S., JørgensenB., *et al*. Mesenchymal stromal cells can be differentiated into endothelial cells *in vitro*. *Stem Cells* 2004;**22**(3):377–384.

148 Nassiri, S.M., Khaki, Z., Soleimani, M., *et al*. The similar effect of transplantation of marrow-derived mesenchymal stromal cells with or without prior differentiation induction in experimental myocardial infarction. *J Biomed Sci* 2007;**14**(6):745–755.

149 Portalska, K.J., Leferink, A., Groen, N., *et al*. Endothelial differentiation of mesenchymal stromal cells. *PLoS ONE* 2012;**7**(10):e46842.

150 Rouhi, L., Kajbafzadeh, A.M., Modaresi, M., *et al*. Autologous serum enhances cardiomyocyte differentiation of rat bone marrow mesenchymal stromal cells in the presence of transforming growth factor-β1 (TGF-β1). *In Vitro Cell Dev Biol Anim* 2013;**49**(4): 287–294.

151 Jallali Tehrani, H., Parivar, K., Ai, J. *et al*. Effect of dexamethasone, insulin and EGF on the myogenic potential on human endometrial stem cell. *Iran J Pharm Res* 2014;**13**(2):659–664.

152 Lionetti, V., Cecchini, M., Ventura, C. Nanomechanics to drive stem cells in injured tissues: insights from current research and future perspectives. *Stem Cells Dev* 2010;**20**(4):561–568.

153 Vittorio, O., Jacchetti, E., Pacini, S., Cecchini, M. Endothelial differentiation of mesenchymal stromal cells: when traditional biology meets mechanotransduction. *Integr Biol* 2013;**5**(2): 291–299.

154 König, J., Huppertz, B., Desove, G., *et al*. Amnion-derived mesenchymal stromal cells show angiogenic properties but resist differentiation into mature endothelial cells. *Stem Cells Dev* 2011;**21** (8):1309–1320.

155 Davani, S., Marandin, A., Mersin, N., *et al*. Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* 2003;**108**(10 suppl 1): II-253–II-258.

156 Lee, M.-Y., Huang, J.-P., Chen, Y.-Y., *et al*. Angiogenesis in differentiated placental multipotent mesenchymal stromal cells is dependent on integrin $\alpha_5\beta_1$. *PLoS ONE* 2009;**4**(10):e6913.

157 Alaminos, M., Pérez-Köhler, B., Garzón, I., *et al*. Transdifferentiation potentiality of human Wharton's jelly stem cells towards vascular endothelial cells. *J Cell Physiol* 2010;**223**(3):640–647.

158 Corotchi, M.C., Popa, M.A., Remes, A., *et al*. Isolation method and xeno-free culture conditions influence multipotent differentiation capacity of human Wharton's jelly-derived mesenchymal stromal cells. *Stem Cell Res Ther* 2013;**4**:81.

159 Crisan, M. Transition of mesenchymal stem/stromal cells to endothelial cells. *Stem Cell Res Ther* 2013;**4**(4):1–3.

160 Mayer, H., Bertram, H., Lindenmaier, W., *et al*. Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stromal cells: autocrine and paracrine role on osteoblastic and endothelial differentiation. *J Cell Biochem* 2005;**95**(4):827–839.

161 Shabbir, A., Zisa, D., Suzuki, G. Lee, T. Heart failure therapy mediated by the trophic activities of bone marrow mesenchymal stromal cells: a noninvasive therapeutic regimen. *Am J Physiol Heart Circ Physiol* 2009;**296**(6):H1888–H1897.

162 Barker, T.H. The role of ECM proteins and protein fragments in guiding cell behavior in regenerative medicine. *Biomaterials* 2011;**32**(18):4211–4214.

163 Wu, C.C., Chao, Y.C., Chen, C.N., *et al*. Synergism of biochemical and mechanical stimuli in the differentiation of human placenta-derived multipotent cells into endothelial cells. *J Biomech* 2008; **41**(4):813–821.

164 Dong, J.D., Gu, Y.Q., Li, C.M., *et al*. Response of mesenchymal stromal cells to shear stress in tissue-engineered vascular grafts. *Acta Pharmacol Sin* 2009;**30**(5):530–536.

165 Engler, A.J., Sen, S., Sweeney, H.L., Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell* 2006;**126**(4):677–689.

166 Pek, Y.S., Wan, A.C., Ying, J.Y. The effect of matrix stiffness on mesenchymal stem cell differentiation in a 3D thixotropic gel. *Biomaterials* 2010;**31**(3):385–391.

167 Mahmoudi, M., Bonakdar, S., Shokrgozar, M.A., *et al*. Cell-imprinted substrates direct the fate of stem cells. *ACS Nano* **7**(10): 8379–8384.

168 Au, P., Tam, J., Fukumura, D., Jain, R.K. Bone marrow-derived mesenchymal stromal cells facilitate engineering of long-lasting functional vasculature. *Blood* 2008;**111**(9):4551–4558.

169 Carrion, B., Huang, C.P., Ghajar, C.M., *et al*. Recreating the perivascular niche *ex vivo* using a microfluidic approach. *Biotechnol Bioeng* 2010;**107**(6):1020–1028.

170 Schönmeyr, B.H., Soares, M., Avraham, T., *et al*. Vascular endothelial growth factor inhibits bone morphogenetic protein 2 expression in rat mesenchymal stromal cells. *Tissue Eng Part A* 2009;**16** (2):653–662.

171 Li, Y., Zheng, L., Xu, X., *et al*. Mesenchymal stromal cells modified with angiopoietin-1 gene promote wound healing. *Stem Cell Res Ther* 2013;**4**(5):113.

172 Joo, H.J., Kim, H., Park, S.W., *et al*. Angiopoietin-1 promotes endothelial differentiation from embryonic stem cells and induced pluripotent stem cells. *Blood* 2011;**118**(8):2094–2104.

173 Di Bernardini, E., Campagnolo, P., Margariti, A., *et al*. Endothelial lineage differentiation from induced pluripotent stem cells is regulated by microRNA-21 and transforming growth factor β2 (TGF-β2) pathways. *J Biol Chem* 2014;**289**(6):3383–3893.

174 Hong, C.P., Park, J., Roh, T.Y. Epigenetic regulation in cell reprogramming revealed by genome-wide analysis. *Epigenomics* 2011; **3**(1):73–81.

175 Culmes, M., Eckstein, H.-H., Burgkart, R., *et al.* Endothelial differentiation of adipose-derived mesenchymal stromal cells is improved by epigenetic modifying drug BIX-01294. *Eur J Cell Biol* 2013;**92**(2):70–79.
176 Ohtani, K., Vlachojannis, G.J., Koyanagi, M., *et al.* Epigenetic regulation of endothelial lineage committed genes in pro-angiogenic hematopoietic and endothelial progenitor cells. *Circ Res* 2011;**109**(11):1219–1229.
177 Zhou, J., Cheng, M., Liao, Y.H., *et al.* Rosuvastatin enhances angiogenesis via eNOS-dependent mobilization of endothelial progenitor cells. *PLoS ONE* 2013;**8**(5):e63126.
178 Gomes, S.A., Rangel, E.B., Premer, C., *et al.* S-Nitrosoglutathione reductase (GSNOR) enhances vasculogenesis by mesenchymal stromal cells. *Proc Natl Acad Sci U S A* 2013;**110**(8):2834–2839.
179 Ahmed, R.P., Haider, K.H., Shujia, J., *et al.* Sonic Hedgehog gene delivery to the rodent heart promotes angiogenesis via iNOS/netrin-1/PKC pathway. *PLoS ONE* 2010;**5**(1):e8576.
180 Ocarino, N., Boeloni, J., Goes, A.M., *et al.* Osteogenic differentiation of mesenchymal stromal cells from osteopenic rats subjected to physical activity with and without nitric oxide synthase inhibition. *Nitric Oxide* 2008;**19**(4):320–325.
181 Birmingham, E., Niebur, G., McHugh, P.E., *et al.* Osteogenic differentiation of mesenchymal stromal cells is regulated by osteocyte and osteoblast cells in a simplified bone niche. *Eur Cell Mater* 2012;**23**:13–27.
182 Tasso, R., Ulivi, V., Reverberi, D., *et al. In vivo* implanted bone marrow-derived mesenchymal stromal cells trigger a cascade of cellular events leading to the formation of an ectopic bone regenerative niche. *Stem Cells Dev* 2013;**22**(24):3178–3191.
183 Kusumbe, A.P., Ramasamy, S.K., Adams, R.H. Coupling of angiogenesis and osteogenesis by a specific vessel subtype in bone. *Nature* 2014;**507**(7492):323–328.
184 Ramasamy, S.K., Kusumbe, A.P., Wang, L., Adams, R.H. Endothelial Notch activity promotes angiogenesis and osteogenesis in bone. *Nature* 2014;**507**(7492):376–380.
185 Kurpinski, K., Lam, H., Chu, J., *et al.* Transforming growth factor-β and Notch signaling mediate stem cell differentiation into smooth muscle cells. *Stem Cells* 2010;**28**(4):734–742.
186 Yan, J., Tie, G., Wang, S., *et al.* Type 2 diabetes restricts multipotency of mesenchymal stromal cells and impairs their capacity to augment postischemic neovascularization in db/db mice. *J Am Heart Assoc* 2012;**1**(6):e002238.
187 Ruiz, E.J., Oeztuerk-Winder, F., Ventura, J.J. A paracrine network regulates the cross-talk between human lung stem cells and the stroma. *Nat Commun* 2014;**5**:3175.
188 Ye, J., Wu, D., Wu, P., *et al.* The cancer stem cell niche: cross talk between cancer stem cells and their microenvironment. *Tumour Biol* 2014;**35**(5):3945–3951.
189 Borovski, T., De Sousa E Melo, F., Vermeulen, L., Medema, J.P. Cancer stem cell niche: the place to be. *Cancer Res* 2011;**71**(3):634–639.
190 Fessler, E., Dijkgraaf, F.E., De Sousa E Melo, F., Medema, J.P. Cancer stem cell dynamics in tumor progression and metastasis: Is the microenvironment to blame? *Cancer Lett* 2013;**341**(1):97–104.
191 Ben-Batalla, I., Schultze, A., Wroblewski, M, *et al.* Axl, a prognostic and therapeutic target in acute myeloid leukemia mediates paracrine crosstalk of leukemia cells with bone marrow stroma. *Blood* 2013;**122**(14):2443–2452.
192 Kato, I., Niwa, A., Heike, T., *et al.* Identification of hepatic niche harboring human acute lymphoblastic leukemic cells via the SDF-1/CXCR4 axis. *PLoS ONE* 2011;**6**(11):e27042.
193 Mendt, M., Cardier, J.E. Stromal-derived factor-1 and its receptor, CXCR4, are constitutively expressed by mouse liver sinusoidal endothelial cells: implications for the regulation of hematopoietic cell migration to the liver during extramedullary hematopoiesis. *Stem Cells Dev* 2011;**21**(12):2142–2151.
194 Kim, M., Koh, Y.J., Kim, K.E., *et al.* CXCR4 signaling regulates metastasis of chemoresistant melanoma cells by a lymphatic metastatic niche. *Cancer Res* 2010;**70**(24):10411–10421.
195 Margolin, D.A., Silinsky, J., Grimes, C., *et al.* Lymph node stromal cells enhance drug-resistant colon cancer cell tumor formation through SDF-1α/CXCR4 paracrine signaling. 2011;**13**(9):874–876.
196 Kokovay, E., Goderie, S., Wang, Y., *et al.* Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. *Cell Stem Cell* 2010;**7**(2):163–173.
197 Plane, J.M., Andjelkovic, A.V., Keep, R.F., Parent, J.M. Intact and injured endothelial cells differentially modulate postnatal murine forebrain neural stem cells. *Neurobiol Dis* 2010;**37**(1):218–227.
198 Gilbertson, R.J., Rich, J.N. Making a tumour's bed: glioblastoma stem cells and the vascular niche. *Nat Rev Cancer* 2007;**7**(10):733–736.
199 Hovinga, K.E., Shimizu, F., Wang, R., *et al.* Inhibition of Notch signaling in glioblastoma targets cancer stem cells via an endothelial cell intermediate. *Stem Cells* 2010;**28**(6):1019–1029.
200 Zhu, T.S., Costello, M.A., Talsma, C.E., *et al.* Endothelial cells create a stem cell niche in glioblastoma by providing NOTCH ligands that nurture self-renewal of cancer stem-like cells. *Cancer Res* 2011;**71**(18):6061–6072.
201 Krishnamurthy, S., Dong, Z., Vodopyanov, D., *et al.* Endothelial cell-initiated signaling promotes the survival and self-renewal of cancer stem cells. *Cancer Res* 2010;**70**(23):9969–9978.
202 Borovski, T., Verhoeff, J.J.C., tenCate, R., *et al.* Tumor microvasculature supports proliferation and expansion of glioma-propagating cells. *Int J Cancer* 2009;**125**(5):1222–1230.
203 Rao, S., Sengupta, R., Choe, E.J., *et al.* CXCL12 mediates trophic interactions between endothelial and tumor cells in glioblastoma. *PLoS ONE* 2012;**7**(3):e33005.
204 Hjelmeland, A.B., Lathia, J.D., Sathornsumetee, S., Rich, J.N. Twisted tango: brain tumor neurovascular interactions. *Nat Neurosci* 2011;**14**(11):1375–1381.
205 Bulnes, S., Bengoetxea, H., Ortuzar, N., *et al.* Angiogenic signalling pathways altered in gliomas: selection mechanisms for more aggressive neoplastic subpopulations with invasive phenotype. *J Signal Transduct* 2012;**2012**:597915.
206 Pham, L.-D.D., Hayakawa, K., Seo, J.H., *et al.* Crosstalk between oligodendrocytes and cerebral endothelium contributes to vascular remodeling after white matter injury. *Glia* 2012;**60**(6):875–881.
207 Teng, H., Zhang, Z.G., Wang, L., *et al.* Coupling of angiogenesis and neurogenesis in cultured endothelial cells and neural progenitor cells after stroke. *J Cereb Blood Flow Metab* 2007;**28**(4):764–771.
208 Salsman, V.S., Chow, K.K.H., Shaffer, D.R., *et al.* Crosstalk between medulloblastoma cells and endothelium triggers a strong chemotactic signal recruiting T lymphocytes to the tumor microenvironment. *PLoS ONE* 2011;**6**(5):e20267.

209 Lozito, T.P., Tuan, R.S. Mesenchymal stromal cells inhibit both endogenous and exogenous MMPs via secreted TIMPs. *J Cell Physiol* 2011;**226**(2):385–396.

210 Gruber, R., Kandler, B., Holzmann, P., *et al.* Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. *Tissue Eng* 2005;**11**(5–6):896–903.

211 Traktuev, D.O., Merfeld-Clauss, S., Li, J., *et al.* A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res* 2008;**102**(1):77–85.

212 Baker, A.H., Zaltsman, A.B., George, S.J., Newby, A.C. Divergent effects of tissue inhibitor of metalloproteinase-1, -2, or -3 overexpression on rat vascular smooth muscle cell invasion, proliferation, and death *in vitro*. *TIMP-3 promotes apoptosis. J Clin Invest* 1998;**101**(6):1478–1487.

213 Ries, C., Egea, V., Karow, M., *et al.* MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stromal cells: differential regulation by inflammatory cytokines. *Blood* 2006;**109**(9):4055–4063.

214 Pasquier, J., Thawadi, H., Ghiabi, P., *et al.* Microparticles mediated cross-talk between tumoral and endothelial cells promote the constitution of a pro-metastatic vascular niche through Arf6 up regulation. *Cancer Microenviron* 2014;**7**(1–2):41–59.

215 Grange, C., Tapparo, M., Collino, F., *et al.* Microvesicles released from human renal cancer stem cells stimulate angiogenesis and formation of lung premetastatic niche. *Cancer Res* 2011; **71**(15):5346–5356.

216 Hiratsuka, S., Nakamura, K., Iwai, S., *et al.* MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis. *Cancer Cell* 2002;**2**(4):289–300.

217 Jung, T., Castellana, D., Klingbeil, P., *et al.* CD44v6 dependence of premetastatic niche preparation by exosomes. *Neoplasia* 2009; **11**(10):1093–1105.

218 Lis, R., Touboul, C., Mirshahi, P., *et al.* Tumor associated mesenchymal stromal cells protects ovarian cancer cells from hyperthermia through CXCL12. *Int J Cancer* 2011;**128**(3):715–725.

219 Corcoran, K.E., Trzaska, K.A., Fernandes, H., *et al.* Mesenchymal stromal cells in early entry of breast cancer into bone marrow. *PLoS ONE* 2008;**3**(6):e2563.

220 Rustom, A., Saffrich, R., Markovic, I., *et al.* Nanotubular highways for intercellular organelle transport. *Science* 2004;**303**(5660): 1007–1010.

221 Pasquier, J., Guerrouahen, B.S., Al Thawadi, H., *et al.* Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med* 2013;**11**(1):94.

222 Peinado, H., Alečković, M., Lavotshkin, S., *et al.* Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med* 2012;**18**(6):883–891.

223 Shen, Q., Wang, Y., Kokovay, E., *et al.* Adult SVZ stem cells lie in a vascular niche: a quantitative analysis of niche cell–cell interactions. *Cell Stem Cell* 2008;**3**(3):289–300.

224 Tavazoie, M., Van der Veken, L., Silva-Vargas, V., *et al.* A specialized vascular niche for adult neural stem cells. *Cell Stem Cell* 2008; **3**(3):279–288.

225 Joubin, K., Richardson, A., Novoa, N., *et al.* The endothelial cell line bEnd.3 maintains human pluripotent stem cells. *Stem Cells Dev* 2012;**21**(12):2312–2321.

226 Galan-Moya, E.M., Le Guelte, A., Lima Fernandes, E., *et al.* Secreted factors from brain endothelial cells maintain glioblastoma stem-like cell expansion through the mTOR pathway. *EMBO Rep* 2011;**12**(5): 470–476.

227 Folkins, C., Man, S., Xu, P., *et al.* Anticancer therapies combining antiangiogenic and tumor cell cytotoxic effects reduce the tumor stem-like cell fraction in glioma xenograft tumors. *Cancer Res* 2007;**67**(8):3560–3564.

228 Daenen, L.G., Shaked, Y., Man, S., *et al.* Low-dose metronomic cyclophosphamide combined with vascular disrupting therapy induces potent antitumor activity in preclinical human tumor xenograft models. *Mol Cancer Ther* 2009;**8**(10):2872–2881.

229 Grellier, M., Granja, P.L., Fricain, J.C., *et al.* The effect of the co-immobilization of human osteoprogenitors and endothelial cells within alginate microspheres on mineralization in a bone defect. *Biomaterials* 2009;**30**(19):3271–3278.

230 Leszczynska, J., Zyzynska-Granica, B., Koziak, K., *et al.* Contribution of endothelial cells to human bone-derived cells expansion in coculture. *Tissue Eng Part A* 2012;**19**(3–4):393–402.

231 Grellier, M., Ferreira-Tojais, N., Bourget, C., *et al.* Role of vascular endothelial growth factor in the communication between human osteoprogenitors and endothelial cells. *J Cell Biochem* 2009;**106** (3):390–398.

232 Liu, Y., Berendsen, A.D., Jia, S., *et al.* Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. *J Clin Invest* 2012;**122**(9):3101–3113.

233 Clarkin, C.E., Emery, R.J., Pitsillides, A.A., Wheeler-Jones, C. Evaluation of VEGF-mediated signaling in primary human cells reveals a paracrine action for VEGF in osteoblast-mediated cross-talk to endothelial cells. *J Cell Physiol* 2008;**214**(2):537–544.

234 Clarkin, C.E., Garonna, E., Pitsillides, A.A., Wheeler-Jones, C. Heterotypic contact reveals a COX-2-mediated suppression of osteoblast differentiation by endothelial cells: a negative modulatory role for prostanoids in VEGF-mediated cell: cell communication? *Exp Cell Res* 2008;**314**(17):3152–3161.

235 Wang, T., Xu, Z., Jiang, W., Ma, A. Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *Int J Cardiol* 2006;**109**(1):74–81.

236 Koninckx, R., Hensen, K., Daniëls, A., *et al.* Human bone marrow stem cells co-cultured with neonatal rat cardiomyocytes display limited cardiomyogenic plasticity. *Cytotherapy* 2009;**11**(6): 778–792.

237 Rajashekhar, G., Traktuev, D.O., Roell, W.C., *et al.* IFATS collection: adipose stromal cell differentiation is reduced by endothelial cell contact and paracrine communication: role of canonical Wnt signaling. *Stem Cells* 2008;**26**(10):2674–2681.

238 James, A.W., Zara, J.N., Zhang, X., *et al.* Perivascular stem cells: a prospectively purified mesenchymal stem cell population for bone tissue engineering. *Stem Cells Transl Med* 2012;**1**(6):510–519.

239 Askarinam, A., James, A.W., Zara, J.N., *et al.* Human perivascular stem cells show enhanced osteogenesis and vasculogenesis with nel-like molecule I protein. *Tissue Eng Part A* 2013;**19**(11–12): 1386–1397.

240 Li, H.-X., Luo, X., Liu, R.-X., *et al*. Roles of Wnt/β-catenin signaling in adipogenic differentiation potential of adipose-derived mesenchymal stromal cells. *Mol Cell Endocrinol* 2008;**291**(1–2):116–124.

241 Campos, M.S., Neiva, K.G., Meyers, K.A., *et al*. Endothelial derived factors inhibit anoikis of head and neck cancer stem cells. *Oral Oncol* 2012;**48**(1):26–32.

242 Neiva, K.G., Zhang, Z., Miyazawa, M., *et al*. Cross talk initiated by endothelial cells enhances migration and inhibits anoikis of squamous cell carcinoma cells through STAT3/Akt/ERK signaling. *Neoplasia* 2009;**11**(6):583–593.

243 Perin, E.C., Tian, M., Marini, III, F.C., *et al*. Imaging long-term fate of intramyocardially implanted mesenchymal stromal cells in a porcine myocardial infarction model. *PLoS ONE* 2011;**6**(9):e22949.

244 Quevedo, H.C., Hatzistergos, K.E., Oskouei, B.N., *et al*. Allogeneic mesenchymal stromal cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci U S A* 2009;**106**(33):14022–14027.

245 Park, H.C., Yasuda, K., Ratliff, B., *et al*. Postobstructive regeneration of kidney is derailed when surge in renal stem cells during course of unilateral ureteral obstruction is halted. *Am J Physiol Renal Physiol* 2010;**298**(2):F357–F364.

246 De Almeida, D.C., Donizetti-Oliveira, C., Barbosa-Costa, P., *et al*. In search of mechanisms associated with mesenchymal stem cell-based therapies for acute kidney injury. *Clin Biochemi Rev* 2013;**34**(3):131–144.

247 Zacharek, A., Chen, J., Cui, X., *et al*. Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC treatment amplifies angiogenesis and vascular stabilization after stroke. *J Cerebr Blood Flow Metab* 2007;**27**(10):1684–1691.

248 Liu, X.B., Jiang, J., Gui, C., *et al*. Angiopoietin-1 protects mesenchymal stromal cells against serum deprivation and hypoxia-induced apoptosis through the PI3K/Akt pathway. *Acta Pharmacol Sin* 2008;**29**(7):815–822.

CHAPTER 27

# The radioresistance of mesenchymal stromal cells and their potential role in the management of radiation injury

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## 27.1 Mesenchymal stromal cells: modulators of hematopoiesis

The bone marrow (BM) is the primary organ in adults in which blood cell formation (i.e., hematopoiesis) occurs. Hematopoiesis is orchestrated by a rare population of pluripotent stem cells, known as hematopoietic stem cells (HSCs). Within the BM, nonhematopoietic stromal cells and various mature immune cells form a specialized microenvironment known as the BM niche. The BM niche provides various developmental cues that instruct HSCs to either remain quiescent (i.e., noncycling), or to undergo cell division and subsequently differentiate into multipotent progenitors. These multipotent progenitors mature into specialized immune cells that comprise innate (e.g., neutrophils, macrophages, and dendritic cells (DCs)) and adaptive (e.g., B and T cells) immunity. Mature immune cells circulate in peripheral lymphoid organs (e.g., the spleen and lymph nodes) and in the blood to exert the various functions of the immune system. Under normal conditions the rates at which mature hematopoietic cells enter and leave the peripheral blood are balanced, a process known as hematopoietic homeostasis. For homeostasis to be achieved, HSC self-renewal and differentiation must be tightly regulated to ensure that mature peripheral blood cells are continuously generated without exhausting the HSC reservoir in the BM. Therefore, tight regulation of HSC self-renewal and differentiation by the BM niche is essential for normal hematopoiesis to be maintained throughout adult life.

The cells that comprise the BM niche are distributed between two compartments: (i) the endosteal niche, located between the bone and the marrow space, and (ii) the perivascular niche, located within the sinusoids of the BM cavity (Figure 27.1) [2–6]; and see Chapter 32.

MSCs are a fundamental component of the BM niche. They are traditionally defined as BM-derived fibroblast-like cells that adhere to plastic and generate colony-forming units (CFU-Fs)

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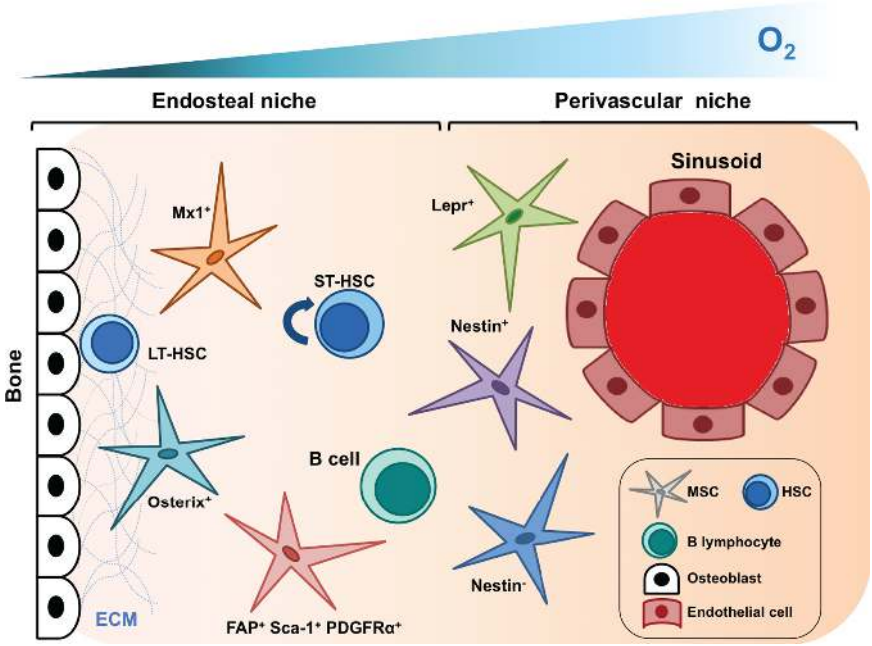



**Figure 27.1** The BM niche. This is segregated into two distinct compartments: (1) the endosteal niche, located proximal to bone, composed of osteoblasts and osteocytes (left); (2) the perivascular niche, located proximal to the vascular sinusoids (right). The BM niche is a hypoxic environment (2–8% $O_2$) with $O_2$ levels gradually decreasing with increasing proximity to the endosteal lining [1,2]. Various mesenchymal stromal cell (MSC) subpopulations of differing lineage potential, defined by specific cell surface marker expression, are distributed throughout the BM niche. These MSC subpopulations secrete a number of extracellular matrix (ECM) components, including cell adhesion molecules, growth factors, and cytokines that regulate defined stages of hematopoiesis, including HSC quiescence, self-renewal, and mobilization and B cell lymphopoiesis. FAP: fibroblast activation protein; LepR: leptin receptor; LT-HSC: long-term or dormant HSC; ST-HSC: short-term or cycling HSC (the gray-scale wedge indicates cycling activity); Mx1: myxovirus resistance protein 1; PDGFRα: platelet-derived growth factor-α; Sca-1: stem cell antigen-1.

*in vitro* [7,8]. They are also multipotent progenitors that differentiate along mesenchymal lineages to become adipocytes (fat cells), osteocytes (bone cells), and chondrocytes (cartilage cells) [9–13]. By flow cytometry, MSCs are distinguished by (1) the lack of hematopoietic cell surface markers such as cluster of differentiation (CD) CD45 (a pan-leukocyte marker), Ter119 (an erythroid marker), and CD3 (a pan T cell marker) and (2) by the presence of cell surface markers that include CD44, CD90, stem cell antigen 1 (Sca-1, in mice), and platelet-derived growth factor receptor (PDGFR)-α [13–15]. However, it is currently unclear whether MSCs express unique cell surface markers. As a result, the identification and isolation of purified MSC populations from the BM remains technically challenging.

Unlike HSCs, the origin(s), functions, and true lineage potential of MSCs in the BM remain poorly understood owing to the technical limitations described. However, the general concept of a stromal cell layer that supports hematopoietic cells *in vivo* coincided with the discovery of the HSC in 1961. In a landmark study by Till and McCulloch, HSCs were indirectly identified by demonstrating quantitative BM transplantation into lethally irradiated mice [16]. Later, hematopoietic colonies were observed in the spleen of reconstituted mice, so-called CFU-Ss, which were initially thought to represent clones of HSCs. However, later studies by Magli *et al.* indicated that CFU-Ss were transient, representing what we call today transient amplifying cells or proliferating precursor cells [17]. These findings also provided the first evidence that a stromal cell compartment capable of supporting hematopoiesis exists *in vivo*. Following this, a variety of MSC lines, such as ST-2, MS5, and OP9, were established *in vitro* from whole BM cultures, to enable the expansion of hematopoietic cells *ex vivo*. The ability to culture purified hematopoietic cell types on these MSC lines *in vitro* has greatly fueled our understanding of hematopoietic cell differentiation, and in particular of B cell development [18–20].

Transgenic Cre reporter mice have become a useful tool for studying specific cell populations *in vivo*. These reporter mice are genetically engineered to encode a fluorescent marker (e.g., green fluorescent protein) that is only expressed upon removal of a STOP codon cassette that is flanked by lox P sites that are recognized and excised by Cre recombinase. Therefore, the use of reporter mice that express Cre recombinase under the control of tissue-specific gene promoters has enabled the identification and lineage tracing of various stem cell types *in vivo*. In recent years, efforts have been made to use such transgenic mice to trace MSCs *in vivo*. These studies have revealed that MSC subpopulations of varying differentiation potential and function coexist within the BM niche. These MSC subpopulations coordinate various stages of hematopoiesis via (1) direct contact with HSCs and their progenitors and (2) the production of various cytokines, growth factors, and ECM molecules. For example, a population of MSC progenitors that express Mx1 has been demonstrated to strictly differentiate osteoblasts *in vivo* and to remodel the BM niche in response to injury [21].

Osteoblasts also closely interact with HSCs in the endosteal niche. Here, osteoblasts secrete ECM components (e.g., N-cadherin and vascular cell adhesion molecule-1), which provide physical support for HSCs. Osteoblasts also secrete various chemokines (e.g., C–X–C motif chemokine ligand (CXCL) 12 – also known as stromal cell-derived factor 1) that regulate HSC self-renewal in the BM [22–24]. CXCL12 production in the

BM is not confined to osteoblasts, however, as several MSC subpopulations that secrete CXCL12 have also recently been identified. CXCL12 production by Osterix-expressing MSCs maintains HSC progenitors and B lymphopoiesis in the endosteal niche [25]. Osterix is a transcription factor involved in osteogenesis. This MSC population also consists of a subset of adipo-osteogenic progenitors known as CXCL12-abundant reticular (CAR) cells. CAR cells produce large amounts of CXCL12 and stem cell factor (SCF) and function to promote HSC self-renewal [25,26]. In addition MSCs that express PDGFR-α, Sca-$1^+$, and FAP-α also produce CXCL12 and SCF and regulate B lymphopoiesis and erythropoiesis in the BM (Figure 27.1) [27,28].

Two distinct populations of MSCs distinguished by the expression of the leptin receptor ($LepR^+$ MSCs) and Nestin ($Nestin^+$ MSCs) have been identified in the perivascular niche (Figure 27.1). Nestin is an intermediate filament protein. These populations function (1) to maintain HSCs within the BM and (2) to suppress HSC mobilization to the spleen in response to injury or stress, a process known as extramedullary hematopoiesis [1,29]. Both $LepR^+$ MSCs and $Nestin^+$ MSCs can differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro*. However, *in vivo* $LepR^+$ MSCs mainly give rise to osteocytes and adipocytes, whereas $Nestin^+$ MSCs have only osteogenic and chondrogenic potential. The discrepancy between the lineage potential of these MSC populations *in vitro* versus *in vivo* suggests that the differentiation potential of MSCs may be altered by growth conditions currently used to expand these cells *in vitro*. Therefore, it will be important to further characterize the differentiation capacity of identified MSC populations *in vivo* to determine the true lineage potential of these MSC subtypes in the physiological setting. Taken together, these findings indicate that interplay between MSC subpopulations and HSCs and their progenitors in the BM niche is likely required for the correct regulation of hematopoiesis *in vivo*.

Oxygen tension is emerging as an important environmental factor that modulates cell behavior, including that of stem cells. Cell lines, including MSC lines, are routinely established and expanded *in vitro* at the inhaled ambient oxygen concentration of 21% $O_2$, commonly referred to as "normoxia." However, the oxygen tension in most tissues is <21%, and therefore these growth conditions are physiologically hyperoxic for a multitude of cell types, including MSCs. The BM niche itself is a relatively hypoxic environment with an estimated oxygen concentration ranging between approximately 0.6% and 8% $O_2$ [2,30,31]. Cellular adaptation to hypoxia is primarily mediated by the transcription factor hypoxia inducible factor-1 (HIF-1). HIF-1 consists of a constitutively expressed HIF-1β subunit and an oxygen-responsive HIF-1α subunit (or their paralogs HIF-2α and HIF-3α). In normoxic conditions prolyl hydroxylase domain-containing proteins (PHDs) hydroxylate proline residues in the HIF-1α degradation domain, enabling the E3 ubiquitin ligase substrate, pVHL (product of the von Hippel-Lindau tumor suppressor), to target HIF-1α for proteasomal degradation. Additionally, factor-inhibiting HIF hydroxylates an asparagine residue in the C-terminal transactivation domain, rendering HIF-1α transcriptionally inactive. Since hydroxylation is dependent on $O_2$ availability, in hypoxic conditions these enzymes no longer posttranslationally modify HIF-1α, leading to HIF-1α stabilization. Stabilized HIF-1α is subsequently translocated to the nucleus, where it dimerizes with β subunits, enabling it to regulate the expression of hypoxia-responsive genes (Figure 27.2) [32–34].

HIF-1α regulates the expression of a myriad of genes involved in metabolism, including growth factor and cytokine production, cell growth and migration, ECM production and, in the DNA damage response, enables cells to functionally adapt to low oxygen environments [32–35]. Studies have demonstrated that, in HSCs, exposure to hypoxia activates transcriptional networks that promote HSC quiescence, differentiation potential, and reconstitutive capacity [2,30,36,37]. Although the effects of hypoxia on MSCs *in vivo* are largely unknown, *in vitro* studies indicate that hypoxia (1) increases the proliferation rate, (2) suppresses senescence, (3) induces pluripotency gene expression and telomerase activity, (4) improves differentiation potential, and (5) alters the cytokine and growth factor profile of MSCs [38–42]. Therefore, it is likely that oxygen tension impacts on MSC and HSC proliferation, differentiation, and function *in vivo*.

## 27.2 The response of mesenchymal stromal cells to ionizing radiation

Exposure to ionizing radiation (IR) (e.g., γ-rays and X-rays) has a multitude of adverse dose-dependent effects on human tissues, including skin burns, hair loss, and shedding of the epithelium lining the gastrointestinal tract. The hematopoietic system is one of the first cellular systems to fail following acute exposure to IR. Lymphocytes in general are highly radiosensitive and die within 24 h following exposure to even low IR doses, whereas granulocytes, erythrocytes, and platelets are more radioresistant [43,44]. HSCs and their progenitors are also radiosensitive, with a $D_0$ of 0.6–1.6 Gy, the dose required to reduce the proportion of surviving cells by 37% less than its previous value, thus preventing continuous renewal of functional blood and immune cell types in the periphery [43,45]. Allogeneic HSC transplantation remains the only practical treatment available for hematopoietic failure following IR exposure. In this process, patients are transplanted with HSCs isolated from a healthy living donor or with umbilical cord blood cells containing HSCs that have been cryopreserved in a cord blood bank. After appropriate pretransplant conditioning, treatment of the recipient (to ablate any residual immune cells that might reject the donor's cells) normal donor HSCs can engraft in the BM of the transplant recipient and reconstitute their hematopoietic and immune systems. Without HSC transplantation, exposed patients will usually die from

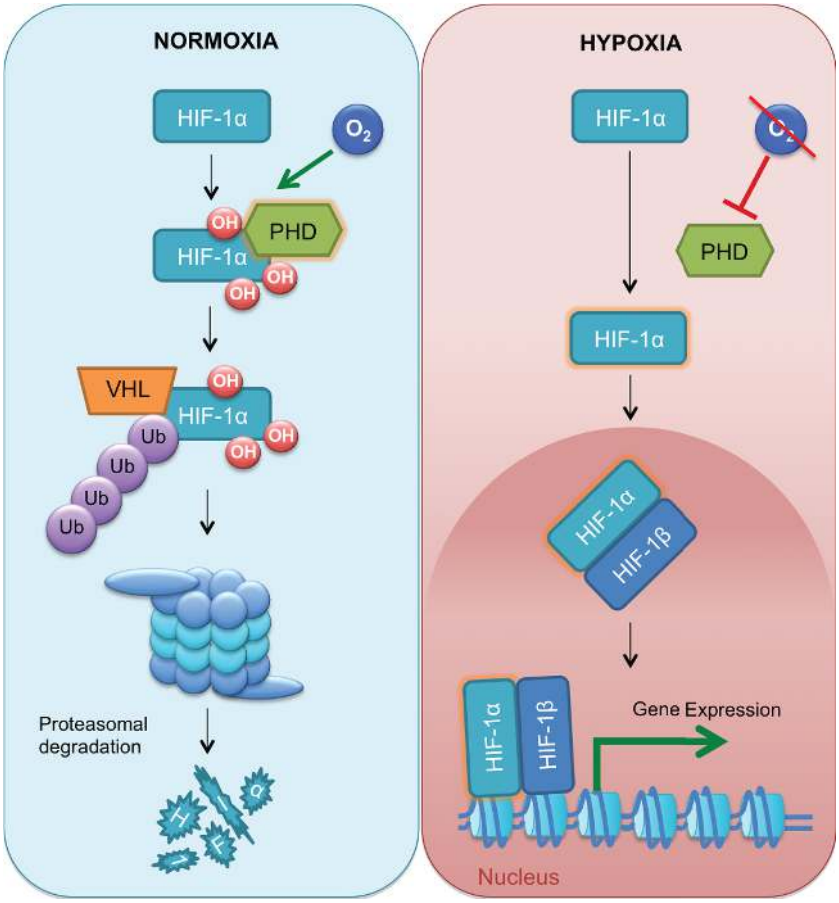



**Figure 27.2** Oxygen-dependent regulation of HIF-1 activity. In normoxia (left panel) the presence of oxygen allows the hydroxylation of HIF-1α by prolyl hydroxylase domain-containing proteins (PHDs), allowing product of the von Hippel–Lindau tumor suppressor (pVHL) to target it for proteasomal degradation. In hypoxia (right panel) the low oxygen levels prevent HIF-1α hydroxylation, allowing its stabilization and translocation to the nucleus, where it dimerizes with HIF-1β and promotes the expression of oxygen-responsive genes. Ub: ubiquitin; OH: hydroxyl groups. (See color plate section.)

complications of hematopoietic failure, which results in anemia, thrombocytopenia, agranulocytosis, and lymphopenia (no red cells, no platelets, no granulocytes (neutrophils), and no lymphocytes) [43,44].

However, unlike hematopoietic cells, MSCs are relatively radioresistant. Studies performed in mice and in humans have demonstrated that MSCs isolated from HSC transplant recipients originate from the irradiated host, rather than from the engrafted donor HSCs [46–51]. In addition MSCs isolated from these recipients can subsequently be expanded *in vitro* to generate cell lines such as MS-5, which (1) retain mesenchymal differentiation capacity, (2) continue to support hematopoiesis *in vitro*, and (3) do not give rise to tumors when injected into mice [46–51]. These findings demonstrate that MSCs within the BM can survive doses of irradiation that are detrimental to the hematopoietic system.

## 27.3 The DNA damage response

The maintenance of genomic stability is essential for cell survival and for preventing malignant transformation. The genome of the cell is continuously challenged by both extrinsic and intrinsic genotoxic stresses that damage DNA, such as environmental mutagens (e.g., IR and ultraviolet light) and reactive metabolic byproducts (e.g., reactive oxygen intermediates). The cellular responses to IR are mostly due to its destructive effects on the genome [52,53]. In general, IR-induced DNA damage can arise (1) from energy deposited directly on to DNA or (2) indirectly from free radicals generated by the ionization of other molecules, such as oxygen and water, that modify and/or break chemical bonds within DNA strands [54]. Cells have evolved a complex signaling network that senses DNA damage and they activate biological responses that promote cell survival or, if the DNA damage present in the cell is too extensive, can stimulate the cell to die. These pathways are collectively known as the DNA damage response (DDR). Conceptually, the DDR consists of (1) sensor proteins that monitor DNA for structural abnormalities such as DNA breaks; (2) transducer proteins that transmit and amplify the DNA damage signal; and (3) effector proteins that orchestrate the desired biological response(s) following the triggering of the DDR [55,56]. These pathways can ultimately lead to either a temporary or permanent halt in cell growth and division, the activation of DNA repair mechanisms and/or the activation of cell death mechanisms (Figure 27.3). Therefore, the activation of specific DDR pathways ultimately determines whether a damaged cell will survive or die in response to genotoxic stress.

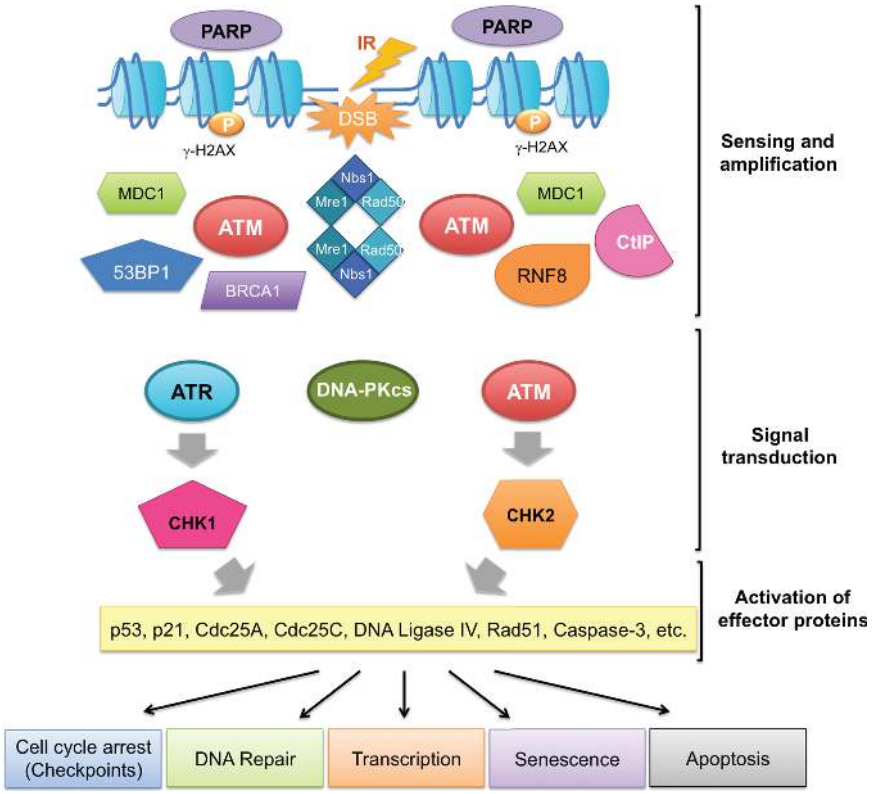



**Figure 27.3** The DDR is composed of sensor, transducer, and effector proteins whose activities induce biological responses to DNA damage. DNA double-strand breaks (DSBs) are initially sensed by poly-(ADP-ribose) polymerase (PARP) and the Mre11–Rad50–Nbs1 (MRN) complex, resulting in the recruitment and activation of the phosphoinositide-3-kinase (PI3K)-related protein kinases, ataxia–telangiectasia-mutated (ATM), and DNA-PKcs (DNA-dependent protein kinase, catalytic subunit – see text for details). Posttranslational modifications to histones surrounding DNA DSBs, including ATM-dependent H2AX Ser139 phosphorylation (γ-H2AX), stimulate the recruitment of DDR mediators, including mediator of DNA damage checkpoint protein 1 (MDC1), p53 binding protein 1 (53BP1), breast cancer type 1 (BRCA1), C-terminus interacting protein (CtIP), and E3 ubiquitin ligase RING finger protein 8 (RNF8), which act to amplify the DDR signal and to modulate ATM-dependent phosphorylation of target substrates. ATM-mediated DNA DSB resection to generate single-stranded DNA (ssDNA) tails also results in ATM and RAD3 related (ATR) activation. Activated ATM and ATR phosphorylate and activate the DDR transducers checkpoint kinase (CHK)2 and CHK1 respectively, which relay the damage signal downstream to effector proteins involved in various biological responses. These include p53 and p21: DNA damage checkpoints, DNA damage-induced transcription, DNA repair, apoptosis, and senescence; Cdc25a: DNA damage checkpoints; DNA ligase IV and Rad51: DNA DSB repair; caspase-3: apoptosis. See text for details.

The most genotoxic lesions caused by IR are DNA DSBs. These can arise when breaks in sister DNA strands occur either directly opposite or within a few base pairs of each other, causing the chromatin to be segregated into two pieces. The lack of a DNA template at the damage site renders these lesions difficult to repair, increasing cellular susceptibility to chromosomal instability and to the induction of cell death mechanisms [54]. It is estimated that each gray of IR introduces 33 DSBs into the mammalian genome [57]. DNA DSBs can also occur endogenously due to telomere uncapping or during DNA replication as a result of the collapse of a stalled replication fork. Replication fork stalling can occur due to presence of various lesions, including single base lesions, cross-links, and single DNA strand breaks (SSBs). It can also occur at natural replication pause sites known as fragile sites, which are frequently associated with chromosome breakage and genomic rearrangements [58–60]. Despite being a major threat to the genome, DNA DSBs also have defined biological purposes and are introduced deliberately, in a well-controlled manner, during meiotic recombination. In developing T and B lymphocytes, DNA DSBs are also induced during V(D)J recombination and class switch recombination, and are essential for generating the extensive repertoire of immunoglobulins and T cell receptors expressed on B cells and T cells respectively, which are central to the adaptive immune response [61,62].

The DDR is primarily regulated by the PI3K-related protein kinase family, which includes ATM, ATR, and DNA-PKcs [54,63–65]. All members share a common PI3K domain that phosphorylates a serine or threonine residue that is followed by glutamine (i.e., an SQ or TQ motif) in target substrates in order to transduce DNA damage signals to various transducer and effector proteins [64]. ATM is the master regulator of DNA DSB signaling pathways, whereas DNA-PKcs are primarily involved in the DNA DSB repair pathway or nonhomologous end-joining (NHEJ), which will be discussed later in further detail. ATR is primarily activated by ssDNA structures that are generated by a range of DNA lesions, including base adducts, cross-links, or single-strand breaks. Such DNA lesions are generated naturally during DNA replication and artificially by various agents that inhibit DNA replication, such as ultraviolet

light and hydroxyurea [58–60,63]. We will mainly focus on the branches of the DDR that mediate the cellular response to DNA DSBs as they represent the most deleterious DNA lesions caused by IR.

### 27.3.1 Sensing damage: DNA damage response initiation

The DDR is activated when DNA lesions such as DNA DSBs are recognized by sensor proteins. DNA DSBs are initially recognized by members of PARP family of proteins. Of the 17 members of the PARP family, only PARP1–3 have so far been demonstrated to be involved in the DDR, with each member having a different specificity. PARP1 is activated by multiple types of DNA lesions, including SSBs, DNA cross-links, stalled replication forks, and DSBs. PARP2 is thought to recognize gaps and flap structures, while PARP3 responds selectively to DNA DSBs, most likely in cooperation with PARP1 [53,66,67]. PARPs catalyze the addition of poly-(ADP) ribose (PAR) chains (i.e., PARylation) to histone tails flanking DNA DSBs. These chains provide a platform for recruiting the MRN complex, which forms a physical bridge between DNA DSB ends and acts as a primary sensor of DNA DSBs [66,68]. The MRN complex is a hexameric structure consisting of dimers of each of its three subunits. The Mre11, Rad50, and Nbs1 subunits collectively function to initiate the DDR, to stimulate the activation of downstream signaling pathways, and also to aid in the repair of DNA DSBs. Mre11 is a U-shaped nuclease that binds to short 3′OH overhangs at DNA DSB termini to form synaptic DNA complexes. Rad50 contains an adenosine-5′-triphosphate-binding cassette that binds and unwinds DNA DSB termini. While Mre11 facilitates short-range synapsis of the two ends at a DNA DSB, the current model suggests that Rad50 mediates long-range tethering of two different DNA molecules; for example, a broken chromosome and its sister chromatid. Nbs1 acts as a flexible adaptor between Mre11 and Rad50 and mediates the assembly and nuclease activity of MRN complexes at DNA DSB sites [69–72]. Recruitment of the MRN complex to DNA DSBs is essential for activating DDR signaling pathways.

As previously mentioned, the PI3K kinases ATM, ATR, and DNA protein kinase, catalytic subunit (DNA-PKcs) are key regulators of DDR signaling. ATM is rapidly recruited to DNA DSBs by the MRN complex and physically interacts with Nbs1. Interaction with the MRN complex stimulates ATM to be posttranslationally modified on multiple residues that (1) activate (e.g., S1981 autophosphorylation) and (2) modulate its function (e.g., TIP60 acetyltransferase-dependent K3016 acetylation). The components of the MRN complex itself are subsequently phosphorylated by activated ATM, which is thought to create a positive feedback loop in order to maintain ATM activity [61,69–76].

### 27.3.2 Sending an SOS: DNA damage response signal transduction and amplification

Following recruitment of PARPs, the MRN complex, and ATM to DNA DSBs, the surrounding chromatin is remodeled in order to amplify the DDR signal downstream. DNA compaction in "closed" heterochromatin obstructs DNA metabolism and repair. Therefore, a more "open" state of chromatin (i.e., euchromatin) is desirable in order to facilitate DNA repair processes. Consequently, immediately following DNA damage, chromatin surrounding DNA DSBs undergoes an energy-dependent local expansion, mediated by adenosine-5′-triphosphate-dependent chromatin remodelers (such as PARP1), in order to facilitate the recruitment of multiple DDR factors to damaged sites [77–79]. During the chromatin remodeling process histones are also posttranslationally modified in order to create docking sites for a family of BRCA1 C-terminal (BRCT)-domain-containing DDR scaffold or "mediator" proteins. DDR mediators are essential for (1) retaining sensor proteins at DNA DSB sites; (2) modulating ATM activation and function; and (3) facilitating DNA repair. DDR mediators are being identified on a continuing basis and include MDC1, RNF8, 53BP1, and BRCA1 [53,80–83].

A well-characterized histone modification generated in response to IR-induced DNA DSBs is γ-H2AX. The γ-H2AX modification is an important signal for DDR activation in response to DNA DSBs and is generally recognized as a marker of DNA DSBs [84–87]. γ-H2AX combined with Tyr143 dephosphorylation (via eyes absent homolog 1/3 [EYA1/3] phosphatases) facilitates the docking of MDC1 on to chromatin at DNA DSB sites where it interacts with ATM and Nbs1. This stimulates the recruitment of additional ATM–MRN complexes and further ATM activation, resulting in H2AX phosphorylation over several megabases (Figure 27.3) [61,88]. Other histone modifications, – for example, acetylation, ubiquitination, and methylation – also stimulate the recruitment of other DDR mediators, such as 53BP1 and BRCA1. This complex assembly of DDR sensors and mediators at DNA DSBs results in the formation of supramolecular structures known as DNA DSB-associated foci, or IR-induced foci (IRIF), which act to amplify the DDR signal downstream and to facilitate DNA DSB repair [68,88–90].

Crosstalk between DDR mediators and ATM at DNA DSBs enables ATM to phosphorylate numerous target substrates, including the DDR transducer Chk2. To facilitate DNA repair, various endonucleases are recruited to DNA DSBs to generate ssDNA tails, a process known as DNA DSB resection. As previously mentioned, ssDNA structures at damage sites are normally recognized by ATR. During DNA DSB resection, ATM mediates replication protein A (RPA) coating of ssDNA tails, which subsequently triggers ATR activation and ATR-dependent phosphorylation of another DDR transducer, Chk1 [65,91–93]. Thus, the DDR is modulated by a crosstalk between ATM–Chk2 and ATR–Chk1 pathways, which together transmit the damage signal to multiple effector proteins that orchestrate biological outcome(s) to DNA DSBs. These outcomes include transient delays in cell cycle progression (termed DNA damage checkpoints), DNA DSB repair, apoptosis, and senescence (Figure 27.3). These processes will now be discussed in further detail.

### 27.3.3 DNA damage checkpoints

The cell cycle comprises the series of events that enable the replication of the genetic material and its subsequent segregation into two new cells. It is composed of four discrete phases: mitosis (M), gap 1 ($G_1$), DNA synthesis (S), and gap 2 ($G_2$). $G_0$ refers to cells that are quiescent (i.e., out of cycle). These are detailed as follows: (1) $G_1$, in which the cell is stimulated to increase in mass by extracellular mitogens and growth factors and prepares for DNA synthesis; (2) S phase, in which DNA synthesis occurs; (3) $G_2$, in which the cell continues to increase in mass and prepares for mitosis; and (4) M phase, in which the cell segregates its newly copied chromosomes and divides [94,95]. Cell cycle progression is driven by a series of protein kinases known as cyclin-dependent kinases (CDKs). The activity of each CDK is regulated by the binding of specific cyclins whose expression levels fluctuate throughout the cell cycle, allowing different CDKs to be only active during specific cell cycle phases. Cyclin D–CDK4/6 and cyclin E–CDK2 complexes promote $G_1$ progression into S phase; cyclin A–CDK2 complexes are required for DNA synthesis and cyclin A– and cyclin B–CDK1 complexes regulate progression through $G_2$ and mitosis (Figure 27.4A). CDK activity is also modulated by (1) phosphorylation on conserved threonine and tyrosine residues by various enzymes, which induce conformational changes that can either promote or inhibit cyclin binding, and (2) by CDK inhibitors (CKIs), which can bind CDKs either alone or in cyclin–CDK complexes, thereby inhibiting CDK activity [52,96–99].

Activation of the DDR signal cascade can induce transient delays at specific cell cycle transitions (such as $G_1$-to-S phase or $G_2$-to-M) or during phases (intra-S phase;, i.e., during DNA replication), and these are known as the DNA damage checkpoints (Figure 27.4A) [52]. These checkpoints function as regulatory systems that ensure all processes in the cell required to replicate the genome are correctly completed before allowing subsequent division into two new daughter cells. In the presence of genotoxic or other cellular stresses, these checkpoints are activated to provide time for pathways required for the resolution of this stress to be activated.

#### 27.3.3.1 $G_1$/S checkpoint

Cells need to ensure that the genome is stable before committing to cell cycle entry. Therefore, the $G_1$/S checkpoint functions to prevent the initiation of DNA synthesis (i.e., entry into S phase) in the presence of DNA damage. The $G_1$/S checkpoint can be activated by ATM- and ATR-dependent phosphorylation of the DDR signal transducers, Chk1 and Chk2. Activated Chk1 and Chk2 in turn target two critical effectors operating in distinct branches of the $G_1$/S checkpoint, the Cdc25a phosphatase and the transcription factor p53. Cdc25a phosphatase activates CDK2, which is involved in the loading on to chromatin of Cdc45, a protein necessary for the activation of prereplication complexes during the initiation of DNA replication. In response to DNA DSBs, ATM-dependent Chk2 phosphorylation of Cdc25a targets it for ubiquitin-dependent proteasomal degradation, preventing

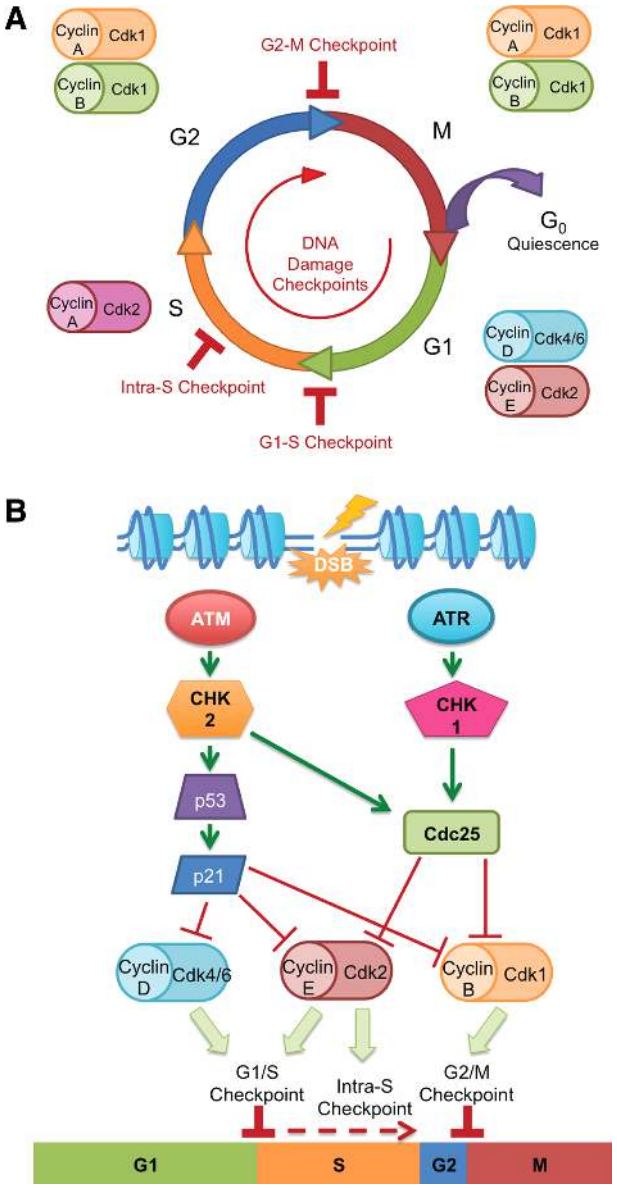



**Figure 27.4** DNA damage checkpoints. (A) The cell cycle is composed by four discrete phases: $G_1$, S, $G_2$, and M. The transition from one phase to the next is controlled by different cyclins and CDKs. These transitions can be delayed by the DDR in response to DNA damage through the activation of specific mechanisms known as DNA damage checkpoints, which are named according to the cell cycle transition that they affect: $G_1$/S, intra-S, or $G_2$/M checkpoints. (B) In response to DNA DSBs, ATM and Chk2 activation leads to downstream p53 stabilization and subsequent induction of p21 transcription. p21 is a potent CDK inhibitor that prevents the activation of cyclin D–Cdk4/6, cyclin E–Cdk2 and cyclin B–Cdk1 complexes, resulting in (1) a block in S-phase entry (i.e., the $G_1$/S checkpoint); (2) a reduction in the rate of DNA synthesis (i.e., the intra-S-phase checkpoint); and (3) a block in mitotic entry (i.e., the $G_2$/M checkpoint). Both ATM–Chk2 and ATR–Chk1 signaling pathways lead to the phosphorylation and targeted degradation of Cdc25a and Cdc25c phosphatases. Cdc25a and Cdc25c degradation prevent the downstream activation of cyclin E–Cdk2 and cyclin B–Cdk1 complexes respectively, inhibiting DNA replication (i.e., the intra-S-phase checkpoint) and blocking entry into mitosis (M) (i.e., the $G_2$/M checkpoint); see text for further details. Cdc: cell division control (protein). (See color plate section.)

CDK2 activation and thereby inhibiting DNA replication. This pathway is rapidly implemented, but is only capable of delaying cell cycle progression for several hours [100–103].

The other branch of the $G_1$/S checkpoint is regulated by the ATM–p53–p21 signaling axis. This pathway is activated slowly as the transcription and translation of downstream proteins are required to induce prolonged $G_1$ arrest. p53 and p21 are two key tumor suppressors that inhibit the proliferation of genetically unstable cells by regulating the expression of genes involved in multiple DDR mechanisms, including cell cycle arrest, DNA repair, and apoptosis. Under normal conditions, p53's function is constitutively maintained at low levels by the E3 ubiquitin ligase, mouse double minute 2 (Mdm2), which targets p53 for proteasomal degradation. However, in response to DNA DSBs, p53 and Mdm2 become posttranslationally modified by various DDR proteins, including ATM and Chk2, leading to p53 stabilization and accumulation. This results in an increase in its transcriptional activity. p21, a CKI, is the key effector of p53-mediated $G_1$ arrest. p53 positively regulates the expression of the CKI, p21, which inhibits (1) cyclin D–CDK4/6 and cyclin E–CDK2 complexes required for the $G_1$/S transition and (2) proliferating cell nuclear antigen (PCNA) activity required for DNA replication, thereby blocking S-phase entry (Figure 27.4B) [52,100–107].

#### 27.3.3.2 Intra-S-phase checkpoint

The intra-S-phase checkpoint is activated in response to replication stress and DNA damage. It is characterized by a reduction in the rate of DNA synthesis. This occurs due to the inhibition of late origin firing, caused by the presence of replication inhibitors and by DNA damaging agents. This checkpoint is also instigated as a result of replication fork stalling, which occurs due to an arrest in DNA elongation caused by the failure of replication complexes to bind to damaged template DNA. This checkpoint functions to protect stalled replication forks from aberrant recombination, promoting the relaxation of chromosomal torsional stress and the completion of replication via upregulation of deoxyribonucleotide (dNTP) production [108]. There are likely two effector cascades that contribute to the IR-induced intra-S-phase checkpoint. The blockade of replication origin firing in response to DNA DSBs is primarily mediated by the previously described ATM–Chk2–Cdc25a signaling axis (Figure 27.4B) [109,110]. The mechanism(s) underlying replication fork stalling in response to DNA DSBs is currently poorly understood. However, cohesin, a protein complex consisting of Structural Maintenance of Chromosome (SMC)1, SMC3, and double-strand-break repair protein RAD21 (known as Sister Chromatid Cohesion protein 1 (SCC1)) that forms a ring around sister chromatids as they are being synthesized during DNA replication, has emerged as an important substrate of ATM signaling in this process. The pairing of sister chromatids, (i.e., cohesion) is essential for accurate chromosome condensation and segregation in mitosis and for DNA DSB repair by homologous recombination (HR) [111,112]. Nbs1–BRCA1–ATM-dependent phosphorylation of SMC1 and ATM–Chk2-dependent phosphorylation of SMC3 are known to be required for replication fork stalling in response to DNA DSBs [113,114]. The molecular mechanism(s) linking ATM-mediated posttranslational modification of cohesin and replication fork stalling remains to be determined.

#### 27.3.3.3 $G_2$/M checkpoint

The $G_2$/M checkpoint prevents cells from initiating mitosis in the presence of DNA damage. This process is essential for preventing chromosomal instability and for ensuring that potentially harmful mutations are not inherited by daughter progeny. In response to DNA DSBs, ATM and Chk2 phosphorylate Cdc25c phosphatase, targeting Cdc25c for proteasomal degradation. As a result, Cdc25c does not remove inhibitory threonine 14/tyrosine 15 phosphorylations present on CDK1, preventing activation of CDK1–cyclin B complexes, thereby blocking entry into mitosis. ATR–Chk1-dependent phosphorylation and subsequent degradation of Cdc25C in response to resected DNA DSBs also contributes to the activation of $G_2$/M arrest in response to resected DSBs as described earlier (Figure 27.4B) [115,116]. The $G_2$/M checkpoint is maintained by two MDC1–53BP1-dependent pathways, depending on the nature of the DNA DSB. 53BP1 and MDC1 sustain activated ATM–Chk2 signaling in response to persisting DNA DSBs, whereas they promote the activation of ATR–Chk1 signaling in response to resected DNA DSBs [116,117]. Overall, activation of DNA damage checkpoints is critical to provide time for persisting DNA damage within the cell to be repaired before undergoing cell division.

## 27.4 DNA double-strand break repair

As previously discussed, DNA DSBs are extremely destructive for the genome. Therefore, failure to repair DNA DSBs, or their misrepair, can result in chromosomal aberrations and subsequently cell death or cell transformation and malignancy. Cells have evolved DNA repair pathways to effectively resolve DNA lesions in order to prevent these biological outcomes. DNA DSBs are primarily repaired via two distinct pathways, known as HR and NHEJ. HR requires a complementary DNA strand within a sister chromatid as a homologous template for DNA DSB repair, whereas NHEJ consists of tethering broken DNA ends together followed by their religation. The requirement of a sister chromatid for repair by HR results in this pathway being restricted only to S and $G_2$ phases of the cell cycle, whereas NHEJ can be activated throughout the cell cycle (Figure 27.5). Therefore, NHEJ is responsible for the repair of the majority of DNA DSBs, while only approximately 15–20% DNA DSBs are repaired via HR [118–121].

### 27.4.1 Nonhomologous end joining

NHEJ is initiated by the binding of each DNA end at the DSB by the ring-shaped Ku70–Ku80 heterodimeric complex.

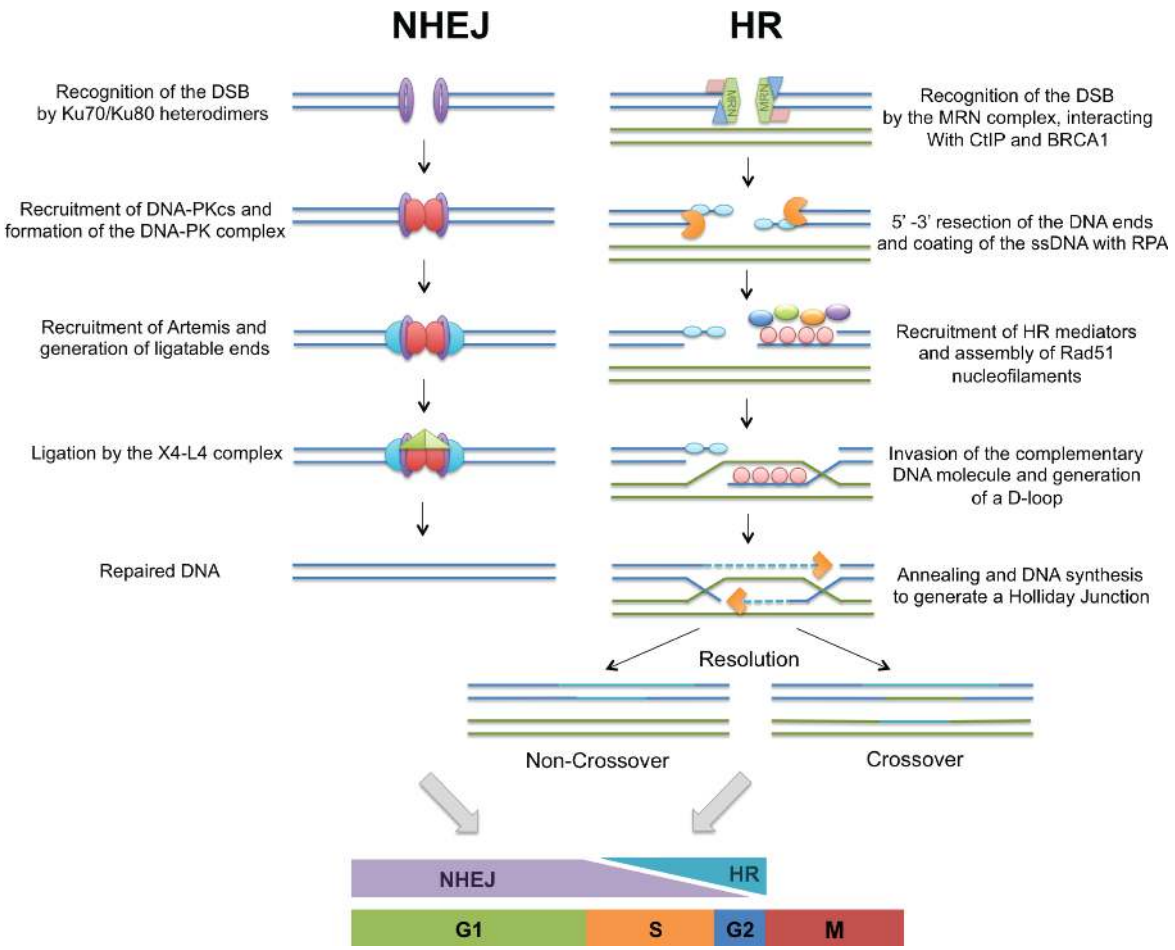



**Figure 27.5** DSB repair mechanisms. In NHEJ (left panel), DNA DSBs are recognized by the Ku70/Ku80 complex that forms a synaptic complex with DNA DSB termini and recruits DNA-PKcs to form the DNA-PK holoenzyme. DNA-PKcs is activated and the 5′–3′ endonuclease Artemis is recruited. Subsequent processing of DNA DSB ends facilitates the recruitment of the X4–L4 complex in which DNA ligase IV is activated and ligates the processed DNA DSB ends, resolving the DSB. In HR (right panel) DNA DSBs are resected by the 5′–3′ endonuclease activity of the MRN/–CtIP–BRCA1 complex, generating ssDNA tails that are subsequently bound by RPA. RPA unwinds DNA secondary structure, facilitating further resection and the recruitment of HR proteins, including BRCA2, X-ray repair cross-complementing protein (XRCC)3, Rad52, and Rad54. These proteins assemble Rad51 molecules along ssDNA tails, generating a Rad51 nucleofilament that displaces RPA oligomers. The Rad51 nucleofilament invades the sister chromatid and facilitates complementary binding between the resected DNA strand (generated at the DNA DSB) and the unprocessed sister DNA strand, resulting in the formation of a displacement (D) loop DNA intermediate. Subsequent annealing of the unresected DNA strand at the DNA DSB to the D loop and DNA synthesis generates a four-stranded DNA structure known as a double Holliday junction. These Holliday junctions can be repaired by either (1) sister chromatid dissociation, forming a non-crossover product or (2) cleavage and subsequent resolution of the cleaved products, generating a crossover product. While NHEJ can take place throughout the entire cell cycle, HR is restricted to S and G2 phases owing to the need for a homologous template (see text for further details). Rad51: a DNA repair protein; X4–L4: a complex consisting of XRCC4, XRCC4-like factor (XLF), and DNA ligase IV.

Ku70–Ku80 heterodimers hold the two ends in close proximity (a process known as synapsis) and recruit DNA-PKcs to DSB termini, together forming the DNA-PK complex in which DNA-PKcs is activated. Depending on the nature of the DNA ends and the complexity of the DSB, different processing factors are subsequently recruited to the DNA DSB. These factors include Artemis (a 5′–3′ endonuclease), polynucleotide kinase, and DNA polymerase X family members that process DNA DSBs to generate 5′ and 3′ ligatable DNA ends. The processed DNA ends are subsequently religated by the X4–L4 complex, consisting of XRCC4, XLF, and DNA ligase IV. XRCC4 and XLF create a scaffold for DNA Ligase IV at the DNA DSB site, resulting in the activation of DNA ligase IV and the resolution of the DNA DSB (Figure 27.5). 53BP1 has emerged as an important mediator and effector in the DDR with roles in DDR initiation, checkpoint signaling, and in DNA repair [119,120,122–125]. Interestingly, 53BP1 has been shown to interact with Rap1-interacting factor (RIF1) at DNA DSBs to block 5′ end resection, thereby promoting DNA DSB repair by NHEJ and inhibiting HR [119,126]. 53BP1 is also involved in long-range end-joining of antigen receptor loci segments in V (D) J recombination and in class switch recombination [127–129].

### 27.4.2 Homologous recombination

Unlike NHEJ, HR employs a complementary DNA strand within a sister chromatid as a homologous template for DNA DSB repair. First, 5′–3′ DNA DSB resection is carried out to create 3′ ssDNA overhangs that are necessary for strand exchange. This process is initiated by the interaction of the MRN complex with CTBP (C-terminus binding protein of adenovirus E1A-interacting protein (CtIP) and BRCA1, and continued by various exonucleases, including Exo1 and the Bloom syndrome helicase. These ssDNA tails are subsequently bound and stabilized by RPA oligomers, which unwind DNA secondary structures, facilitating the recruitment of HR factors. Rad51 recombinase translocates to the nucleus where various mediators, including BRCA2, XRCC3, Rad52, and Rad54, recruit and assemble Rad51 molecules along ssDNA strands, forming a nucleoprotein filament that displaces RPA. BRCA2-dependent loading of Rad51 at processed DNA DSBs results in Rad51 IRIF formation, which can be used experimentally as a marker of DNA DSB repair by HR. The Rad51 nucleoprotein filament then captures the duplex DNA of the intact homologous DNA region in the sister chromatid and invades a single DNA strand, generating a displacement loop intermediate within the sister chromatid. The invasion is followed by extension along both broken DNA strands by a DNA polymerase, most likely Poln, resulting in the formation of a four-stranded DNA structure known as a double Holliday junction. These complex DNA structures are resolved by resolvase complexes, such as Bloom syndrome protein–topoisomerase IIIα complex, resulting in the generation of repaired crossover (reciprocal exchange of DNA sequences) or noncrossover DNA products (Figure 27.5). The type of DNA product generated by HR is tightly regulated. In mitotic cells, the generation of crossover DNA products is disfavored when the DNA template selected fails to locate the identical sister chromatid. This is important for preventing genomic instability, as loss of heterozygosity and translocations can occur due to invasion of the wrong chromosome, and copy number changes may arise as a consequence of inaccurate template selection in repetitive DNA sequence regions such as ribosomal DNA sequences [122,124,130–135].

### 27.4.3 DNA double-strand break repair pathway choice

The choice between NHEJ and HR pathway activation needs to be tightly regulated in order to ensure maintenance of genomic stability. Repair pathway choice is critically influenced by the decision to initiate 5′–3′ DNA DSB end resection, which is now known to be regulated by the DDR mediators 53BP1 and BRCA1. This resection process is necessary for the activation of HR and simultaneous suppression of NHEJ [119,133]. At DNA DSBs, 53BP1 collaborates with RIF1 and paired box transactivation activation domain-interacting protein (PTIP) to inhibit the association of BRCA1 with MRN complex-bound CtIP, preventing DNA DSB resection. Once the cell enters S phase, CtIP is phosphorylated by CDKs, enabling the formation of the MRN–CtIP–BRCA1 complex. This complex displaces 53BP1, RIF1, and PTIP at DNA DSB sites, enabling DNA DSB resection to be initiated, subsequently leading to the activation of HR [119,132–138]. Together, NHEJ and HR enable cells to effectively repair DNA DSBs, contributing to the restoration of genomic integrity and promoting cell survival.

## 27.5 Apoptosis

DNA damage checkpoints and DNA repair mechanisms cooperate to restore genomic stability following genotoxic insult. However, when DNA damage is too extensive within the cell or cannot be repaired, cells can activate mechanisms to ensure that cells harboring potentially harmful genetic mutations are eliminated, or at least lose their proliferative capacity. Apoptosis is a type of programmed cell death that eliminates damaged and stressed cells and is the most prominent type of cell death activated in the presence of DNA damage [139,140]. Apoptosis is characterized by cellular and nuclear shrinkage (pyknosis), chromatin condensation, nuclear fragmentation, blebbing, and the formation of apoptotic bodies. Apoptosis is mainly executed by a family of cysteine proteases known as caspases, which coordinate two main apoptotic signaling pathways known as (1) the intrinsic or mitochondrial pathway and (2) the extrinsic or death receptor-mediated pathway. Caspases are synthesized as inactive zymogens that become functionally active upon cleavage. (A zymogen (or proenzyme) is an inactive enzyme precursor.) Each pathway consists of initiator caspases (e.g., caspase-8, -9, and -10) that cleave and activate executioner caspases (caspase-3, -6, and -7) that act on various proteins involved in multiple biological processes. While the extrinsic apoptotic pathway is initiated by the binding of extracellular signals to death receptors on the cell surface, the intrinsic apoptotic pathway is activated by intracellular stresses including DNA damage [141–144].

The intrinsic apoptotic pathway is regulated by the B cell lymphoma-2 (Bcl-2) family of proteins that are characterized by the presence of one to four conserved Bcl-2 homology (BH) domains. The Bcl-2 family consists of antiapoptotic proteins such as Bcl-2, Bcl-xl, and myeloid cell leukemia 1) and proapoptotic proteins divided into two subfamilies: (1) the BH3-only family, which includes Bid, Bim, Noxa, and Puma; and (2) the Bax family, which includes Bax, Bok, and Bak [141,145–147]. The Bcl-2 family acts as a "life/death" switch that is orchestrated via interactions between the antiapoptotic and proapoptotic members to ensure cell death is activated at the appropriate time and in a well-controlled manner. Under normal conditions, antiapoptotic Bcl-2 family members bind and sequester proapoptotic Bax family members, preventing the induction of apoptosis. In response to DNA DSBs, ATM–Chk2-dependent p53 stabilization can lead to the transcription of the p53 target genes, Puma and Noxa. Although Bim is not a direct transcriptional target of p53, it also plays a key role in activating apoptosis in response to DNA DSBs [148,149]. These activated BH3-only

proteins insert their BH3 domain into the hydrophobic groove of antiapoptotic Bcl-2 proteins, releasing Bax and Bak molecules. Bax, Bak, and other activated BH3-only proteins subsequently oligomerize at the outer mitochondrial membrane, forming pores that facilitate the release of proapoptotic factors from the mitochondrial intermembrane space into the cytosol [141,144–147].

Cytochrome C is one of the most important proapoptotic factors released in this process that interacts with apoptotic protease-activating factor 1 and deoxyadenosine triphosphate in the cytosol, collectively forming the apoptosome. Formation of the apoptosome initiates a signaling cascade in which a sequential series of initiator caspases (e.g., caspase-9) and downstream executioner caspases (e.g., caspase-3) cleave and inactivate target proteins. Thus, executioner caspase-3 can cleave PARP (normally involved in DNA SSB repair) in the presence of extensive DNA damage, preventing PARP-dependent recruitment of other DNA repair proteins to DNA damage sites, ultimately resulting in DNA degradation. Caspase-3 also cleaves and activates the negative regulatory subunit of the caspase-activated DNAse, resulting in DNA cleavage. Cleavage of nuclear lamina results in nuclear shrinking and blebbing, and cleavage of cytoskeletal proteins and cell adhesion molecules contributes to the loss of cell shape. These processes altogether culminate in cellular disassembly and death [144,146,150].

## 27.6 Cellular senescence

Cellular senescence is defined as an irreversible cell cycle arrest that is restricted to proliferating cells. The causes of cellular senescence are still under investigation and include (1) telomere dysfunction, (2) unrepaired DNA damage, (3) oxidative stress, and (4) excessive mitogenic signals, such as overactivation of oncogene expression [151–154]. Senescent cells remain alive and metabolically active, but are characterized by a stable loss of proliferative capacity and altered gene expression that results in multiple cellular changes, including enlarged and defective mitochondria, increased β-galactosidase production in lysosomes, protein aggregation in the endoplasmic reticulum, and an enlarged flattened morphology [154]. In response to DNA DSBs, cellular senescence can be activated by the ATM–Chk2 signaling axis via the phosphorylation and stabilization of p53 [152]. As previously described, in response to DNA damage, p53 can induce the expression of the tumor suppressor p21. p21, in turn, inhibits cyclin D–Cdk4/6, cyclin E–Cdk2, and PCNA activity, halting $G_1$ progression into S phase. The exact mechanisms that underlie the decision to either transiently arrest (i.e., checkpoint) or permanently arrest (i.e., cellular senescence) cell cycle progression at the $G_1$/S transition are poorly understood. However, it is hypothesized that the presence of unrepaired DNA damage within cells may cause p53–p21 signaling to be sustained long term, ultimately leading to permanent cell cycle arrest (i.e., senescence) [151].

Cellular senescence can also be activated via the $p16^{INK4A}$-pRb (retinoblastoma protein) pathway in response to DNA damage. This pathway controls the $G_1$/S phase transition via the regulation of the transcription factor E2F1, whose target genes regulate passage through the restriction point in late $G_1$ and stimulate entry into S phase. $p16^{INK4a}$ is a CKI encoded by the the *ink4a/arf* locus. In response to DNA damage $p16^{INK4a}$ binds to the cyclin D–CDK4/6 complex, preventing it from phosphorylating and inactivating pRb. As a result, pRb remains active and suppresses the activity of E2F1, preventing it from activating target genes required to transit from $G_1$ to S phase, thereby halting cell cycle progression in $G_1$ [99,152,154]. Overall, apoptosis and senescence are key tumor-suppressive mechanisms that prevent the survival and/or growth of cells harboring persistent DNA damage.

## 27.7 Stem cells exhibit a mixed response to DNA damage

The maintenance of genomic integrity in stem cells is indispensable for development and for maintaining tissues throughout adult life. The deleterious effects of DNA damaging agents, such as IR, on tissue viability and regeneration have fueled mounting interest in the DDR of stem cells and their progenitors. Interestingly, these studies have collectively demonstrated that the level of radiosensitivity varies widely between different stem cell types. For example, embryonic stem cells, HSCs, and intestinal stem cells are highly radiosensitive and undergo extensive apoptosis following irradiation (IR) [155–157]. However, other stem cell types, such as keratinocyte stem cells (KSCs) and hair follicle bulge stem cells (BSCs), are relatively resistant to IR-induced cell death [158,159]. Interestingly, radioresistant stem cells such as BSCs and KSCs can repair IR-induced DNA DSBs, whereas radiosensitive stem cells such as HSCs have a poor DNA DSB repair capacity [157,158,160]. IR-induced DNA damage can also stimulate the differentiation of certain stem cell types; for example, (1) of melanocyte stem cells into mature melanocytes and (2) of HSCs into lymphocytes [161,162]. However, the differentiation of other stem cell types, including BSCs and KSCs, is unaffected by irradiation [158,159]. These studies collectively indicate that the response of stem cells to DNA damaging agents is cell type dependent. The individual response of each stem cell type is strongly linked to the differential activation of specific DDR pathways that can either promote survival or induce cell death.

## 27.8 The DNA damage response of mesenchymal stromal cells

Studies to date clearly indicate that MSCs are radioresistant [46–51]. However, the mechanisms that underlie MSC radioresistance are poorly understood. Long-term proliferation

of primary human MSCs *in vitro* following exposure to high-dose irradiation is comparable to that of known radioresistant cell types, such as the A549 lung cancer cell line [163]. Irradiated human MSCs phosphorylate and activate ATM and induce γ-H2AX formation, which is abrogated in the presence of inhibitors that target ATM and DNA-PKcs [163,164]. Thus, the DDR is activated by irradiated human MSCs *in vitro*. The radioresistance of murine MSCs is comparable to that of human MSCs. However, primary mouse MSC cultures exhibit variable induction of γ-H2AX expression and foci formation following irradiation [165]. This suggests that primary mouse MSC cultures exhibit a heterogeneous response to IR-induced DNA damage. Primary MSC cultures can be established from whole BM aspirates previously depleted of hematopoietic cells. However, as described earlier, different stromal cell populations of varying function and differentiation potential are present within the BM. These "'bulk" uncloned MSC cultures are highly heterogeneous, with phenotypic characteristics varying with passage number and also between independent isolations. The inherent heterogeneity of these MSC cultures makes it difficult to extensively characterize the mechanisms that contribute to MSC radioresistance.

To overcome the difficulties presented by using bulk MSC cultures, recent studies have been performed on clonal mouse MSC lines in order to investigate MSC radiobiology in greater detail [165,166]. The radioresistance of the MSC lines MS-5 and ST-2 is comparable to that of primary mouse MSCs [165]. Detailed investigation into the response of these MSC lines to irradiation has demonstrated that several aspects of DDR signaling are likely to contribute to MSC radioresistance. As described previously, γ-H2AX on chromatin surrounding DNA DSBs facilitates the recruitment of various DDR mediator proteins to damage sites, leading to the activation of DDR signaling cascades. Thus, γ-H2AX is a well-defined marker of DDR activation in response to DNA DSBs. The kinetics of γ-H2AX formation were compared between irradiated MSC lines and a radiosensitive immature ($CD4^+$ $CD8^+$) thymocyte line known as ST4.5. These experiments demonstrated that irradiated MSCs induced maximal H2AX phosphorylation and γ-H2AX IRIF formation at a faster rate than radiosensitive ST4.5 thymocytes. ATM and DNA-PKcs function redundantly to phosphorylate H2AX, and chemical inhibition of their kinase activity delays H2AX phosphorylation [167,168]. Interestingly, MSCs were found to endogenously express higher levels of the key DNA DSB response proteins ATM and DNA-PKcs than ST4.5 thymocytes do. Taken together, these findings may indicate that the abundance of important DDR initiator proteins promotes rapid γ-H2AX formation at DNA DSB sites in irradiated MSCs, enabling robust activation of DDR signaling pathways that promote MSC survival.

The resolution of γ-H2AX and/or γ-H2AX foci in irradiated cells over time is an indirect indicator of DNA DSB repair. Following low-dose irradiation (1 Gy), γ-H2AX was resolved at a faster rate in irradiated MSCs than in ST4.5 thymocytes. Furthermore, while γ-H2AX was resolved in MSCs following high-dose irradiation (10 Gy), γ-H2AX persisted long term in irradiated ST4.5 thymocytes. These findings indicate that MSCs have an enhanced ability to repair IR-induced DNA DSBs compared with immature thymocytes [165]. Similar to human MSCs, mouse MSC lines can repair IR-induced DNA DSBs using both NHEJ and HR pathways [163–166]. It has been shown that the majority of IR-induced DNA DSBs are repaired at early time-points after IR, primarily via NHEJ [121]. Similarly, DNA DSBs are largely repaired in MSCs by 4–8 h after high-dose (10 Gy) irradiation [166]. Interestingly, the MSC line MS-5 exhibited an increased capacity to repair DNA DSBs via NHEJ compared with radiosensitive ST4.5 thymocytes [165]. In addition, mouse MSCs expressed higher levels of key DNA DSB repair proteins such as ATM, DNA-PKcs, DNA ligase IV, and Rad51 than ST4.5 thymocytes did. Overall, these findings indicate that MSCs have a robust capacity to repair IR-induced DNA DSBs that is likely promoted by expressing high endogenous levels of key DNA DSB repair proteins.

The presence of DNA damage, including DNA DSBs, can lead to the activation of signaling pathways that results in either delayed cell cycle progression or transient arrest at transitions between cell cycle phases, known as DNA damage checkpoints. In response to DNA DSBs, activation of the $G_1$/S DNA damage checkpoint is primarily mediated via p53-dependent induction of p21, as previously described. Irradiated human MSCs maintain p53 stabilization over time and strongly induce p21 expression after IR, indicating robust activation of the $G_1$/S checkpoint [163,164,169]. However, both primary and clonal murine MSCs only transiently activate the $G_1$/S checkpoint, indicated by (1) transient p53 stabilization and (2) progression of irradiated $G_1$ cells into S phase after high-dose irradiation [165,166]. The lack of a definitive $G_1$/S checkpoint response in mouse MSCs may be correlated with p53 dysfunction in these cultured cells. However, in the presence of persisting DNA DSBs, which can be induced in cells using specific chemotherapeutic drugs such as etoposide, persistent p53 stabilization and downstream induction of p53 target proteins p21 and Puma are readily detectable in mouse MSC lines. Interestingly, transient activation of the $G_1$/S checkpoint has also been reported to occur in irradiated hair follicle BSCs, embryonic stem cells, and human induced pluripotent stem cells [158,169,170]. Like MSCs, BSCs also have a high capacity for repairing IR-induced DNA DSBs via NHEJ and repair DSBs at a similar rate to that of MSCs [158]. Taken together, these findings suggest that the transient activation of the $G_1$/S checkpoint in irradiated mouse MSCs may be correlated with their ability to rapidly resolve DNA DSBs, thereby permitting $G_1$ cells to progress through the cell cycle after irradiation.

Induction of DNA DSBs in S-phase cells can activate the intra-S-phase checkpoint that is characterized by a reduction in the rate of DNA replication, delaying S-phase progression. Cells that accumulate DNA damage during $G_2$ or carry remaining DNA damage from S phase can activate the poorly

understood $G_2$/M checkpoint, which inhibits mitosis in the presence of DNA DSBs.

Activation of the intra-S and $G_2$/M checkpoints can be assessed by monitoring the cell cycle progression of 5′-bromodeoxyuridine (BrdU)-labeled cells. BrdU is a thymidine analog that is incorporated into cells' DNA during DNA replication. Flow cytometric analysis of BrdU-labeled cells with an anti-BrdU antibody and a DNA marker, such as propidium iodide, enables one to distinguish BrdU-labeled S-phase cells from BrdU-unlabeled $G_1$, $G_2$, and mitotic cells. Analysis of BrdU-labeled MSCs demonstrated that both primary and cloned murine MSCs delay S-phase progression and subsequently accumulate in $G_2$ for at least 12 h after high-dose irradiation *in vitro* [165]. These observations indicate that although irradiated mouse MSCs only transiently activate the $G_1$/S checkpoint, the intra-S-phase and $G_2$/M checkpoints are robustly activated by this cell type. The reason(s) why irradiated mouse MSCs preferentially activate intra-S and $G_2$/M checkpoints whereas irradiated human MSCs induce a robust $G_1$/S checkpoint are unclear. It is evident that both mouse and human MSCs can repair DNA DSBs via NHEJ and HR [163–166]. However, whether the capacity of MSCs to repair DNA DSBs by these two pathways is comparable between mouse and human is unknown and may account for the preferential activation of specific DNA damage checkpoints in these cell types.

Importantly, irradiated MSCs can subsequently recover from $G_2$/M arrest and reenter cell cycle. In addition, irradiated MSCs retain the ability to differentiate along mesenchymal-derived lineages [165,171]. These findings indicate that the proliferative capacity and differentiation potential of MSCs remain intact long term after irradiation. However, radiosensitive ST4.5 thymocytes undergo rapid cell death after irradiation, independent of cell cycle phase status. This is due to the rapid activation of apoptosis, indicated by strong p53 stabilization and extensive cleavage of caspase-3 and of PARP in this cell type after irradiation [165]. This phenomenon, previously coined "rapid interphase death," occurs commonly in irradiated lymphocytes. However, unlike ST4.5 cells, and despite harboring intact apoptotic machinery, apoptosis is weakly activated by irradiated mouse MSCs, and cell death mainly only occurs when these cells attempt to reenter the cell cycle following $G_2$/M arrest [165]. In addition, MSCs express high levels of the antiapoptotic proteins Bcl-2 and Bcl-xl, and low levels of the potent proapoptotic proteins Bim and Puma [165]. The intrinsic expression levels of various apoptotic proteins are known to influence the therapeutic sensitivity of various cell types, including HSCs, BSCs, KSCs, and lymphocytes [149,158,172,173]. Therefore, abundant expression of antiapoptotic proteins in MSCs may also enhance their ability to activate prosurvival mechanisms by suppressing the activation of cell death.

Overall, these findings demonstrate that, in response to IR, MSCs preferentially activate DNA damage signaling pathways that promote cell survival. Rapid induction of γ-H2AX in irradiated MSC nuclei, probably promoted by high expression levels of key DDR proteins, is likely to aid MSCs in rapidly initiating protective DNA damage checkpoints and DNA DSB repair. DNA damage checkpoints enable cells to extend the time frame in which DNA damage can be repaired before entering the next phase of the cell cycle. Therefore, coordinated activation of DNA damage checkpoints and DNA repair mechanisms is likely important for efficient resolution of genomic instability in MSCs.

Why MSCs preferentially activate prosurvival pathways in response to DNA damage is unknown. However, mounting evidence indicates that the DDR plays fundamental roles in stem cell biology. The DDR functions in stem cells to monitor endogenous DNA damage caused by environmental factors, such as reactive oxygen species production, and by telomere shortening, preventing stem cell dysfunction and exhaustion *in vivo* [174–177]. In addition, several key DDR proteins, such as ATM, p53, and p21, and DNA DSB repair proteins are also required in stem cells for (1) maintaining self-renewal, (2) inhibiting senescence, and (3) regulating their differentiation potential [160–162,174–180]. Interestingly, proteins involved in genome stability and cell cycle control have also been directly implicated in MSC biology. For example, loss of (1) the cell cycle regulator pRb, (2) the microRNA processor Dicer, and (3) telomerase, which is required for telomere maintenance and stability, impairs the ability of MSCs to regulate hematopoiesis, resulting in myeloproliferation and the development of leukemia [181–183]. In addition, deletion or loss-of-function mutations in several DDR genes, including ATM, ATR, and DNA ligase IV, can result in growth delay, skeletal abnormalities, and osteoporosis [184–186]. Overall, these findings suggest that MSC radioresistance may perhaps be a consequence of the importance of (1) the maintenance of genomic stability for stem cell homeostasis and (2) DDR components in regulating MSC self-renewal, differentiation and function in the BM.

## 27.9 Effects of hypoxia on mesenchymal stromal cell radioresistance

MSCs are radioresistant progenitors that reside in hypoxic niches *in vivo*, particularly in the BM [4,30,31,166,187]. In cancer, hypoxia within tumors and HIF-1α overexpression in tumor cells are associated with increased malignancy and metastatic potential and with poor patient outcome [188,189]. This prognosis is highly correlated with the increased resistance of hypoxic cancer cells to cancer therapies, including radiotherapy [189,190]. Mounting evidence indicates that exposure to hypoxia can alter the DDR of cancer cells, contributing to their increased resistance to DNA-damaging agents, including IR [192,193]. In addition, cellular exposure to severe hypoxia (<1% $O_2$) can activate the DDR independently of DNA damage, indicating that DDR signaling may contribute to adaptation to low oxygen environments [193–197]. Hyperbaric oxygen treatment involves the administration of 100% oxygen above normal atmospheric pressure to increase the amount of dissolved oxygen in

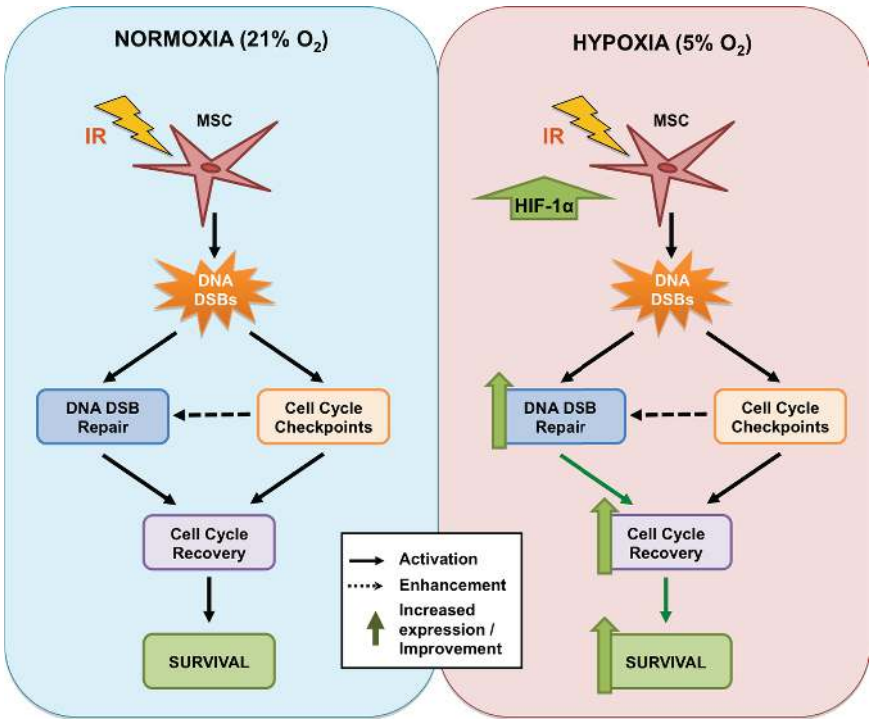



**Figure 27.6** The DDR of mouse MSCs. In normoxia (left panel) and hypoxia (right panel), irradiated MSCs activate DNA damage checkpoints and DNA DSB repair (NHEJ and HR) to resolve genotoxic DNA DSBs. Resolution of DNA DSBs likely enables irradiated MSCs to recover from cell cycle arrest and promotes MSC survival. In hypoxia (right panel), HIF-1α stabilization resulted in (1) increased endogenous expression of the two key NHEJ enzymes, DNA-PKcs and DNA ligase IV, and (2) accelerated recruitment of Rad51 to DNA DSBs, an essential component of repair by HR. Consequently, the DNA DSB repair capacity of MSCs is increased in hypoxia, accelerating recovery from cell cycle arrest and improving long-term survival.

plasma [198]. This treatment has been proposed as a means of increasing oxygen levels within tumors in the hope of augmenting the efficacy of anticancer treatments. However, a recent review indicated that various tumor types respond differentially to the effect of hyperbaric oxygen treatment [198]. Further studies in this area are clearly necessary. Whether exposure to hypoxia affects the resistance of MSCs to irradiation is largely unknown.

In one study, the effect of γ-irradiation on mouse MSCs cultured in normoxia (21% $O_2$) and hypoxia (5% $O_2$) was directly compared. This study demonstrated that both primary mouse MSCs and MSC lines displayed increased resistance to γ-irradiation *in vitro* when cultured in hypoxia [166]. DDR activation, indicated by γ-H2AX formation, was comparable between normoxic and hypoxic MSCs. In addition, MSCs also activated potent intra-S-phase and $G_2$/M checkpoints in hypoxia. However, irradiated MSCs were found to recover from cell cycle arrest at a faster rate in hypoxia than in normoxia [166]. Furthermore, γ-H2AX and γ-H2AX IRIF were resolved at a faster rate in MSC nuclei in hypoxia than with normoxia. Interestingly, MSCs were found to upregulate the expression of key NHEJ repair proteins, including DNA-PKcs and DNA ligase IV in hypoxia. The increased expression of these repair proteins coincided with HIF-1α stabilization in MSCs upon exposure to hypoxia. dsDNA break repairs are reconstituted using proteins of homologous recombination. Rad51 IRIF formation, a key step in the activation of homologous reconstitution, was also accelerated in hypoxic MSCs. Small-interfering-RNA-mediated depletion of HIF-1α in MSCs prevented the upregulated expression of these DNA repair proteins in hypoxic MSCs [166]. These results indicate that HIF-1α may function as a transcriptional modulator of DNA DSB repair in mouse MSCs. Importantly, depletion of HIF-1α rendered MSC lines more radiosensitive. Overall, this study demonstrated that enhanced MSC radioresistance in hypoxia is likely due to alterations in the DDR of MSCs to IR-induced DNA DSBs. These alterations include an enhanced DNA DSB repair capacity that is mediated, at least in part, by the activation of HIF-1α signaling in hypoxic MSCs (Figure 27.6) [166].

Overall, *in vitro* studies to date demonstrate that the DDR plays a major role in mediating the radioresistance of MSCs. Multiple aspects of the DDR are likely to collectively contribute to MSC radioresistance, including (1) rapid activation of DDR signaling, (2) robust DNA damage checkpoints, (3) efficient DNA DSB repair, and (4) suppression of apoptosis (Figure 27.6). The ability to restore the hematopoietic system of an irradiated mouse or human by transplanting donor HSCs into the recipient BM microenvironment is strong evidence that MSCs are also resistant to irradiation *in vivo*. However, as previously described, the BM niche consists of several MSC subpopulations of varying function, proliferative capacity, and differentiation potential. Therefore, it is likely that individual MSC subpopulations exhibit differential DDR signaling responses to IR-induced DNA damage. Mouse MSCs isolated from flushed BM aspirates (BM-MSCs) were reported to be more radiosensitive than MSCs isolated from collagenase-digested bone [199]. Interestingly, $p16^{INK4A}$ tumor suppressor protein was expressed at higher levels in irradiated MSCs from

collagenase-digested bone than in BM-MSCs, whereas $p19^{ARF}$ expression levels were only increased in irradiated BM-MSCs. Furthermore, BM-MSC numbers were significantly reduced in irradiated mouse femurs, and this reduction was rescued in *ink4a/arf*-deficient mice, whereas MSC numbers from collagenase-digested bone were unaffected by irradiation [199]. In addition, human MSCs derived from maxillary and mandibular trabecular bones (orofacial MSCs) were reported to be more radioresistant than MSCs derived from the iliac crest [169]. Orofacial bone MSCs induced higher p21 expression and harbored less DNA damage than iliac crest MSCs after IR treatment. Furthermore, injection of irradiated MSCs into mice demonstrated that bone formation by iliac crest MSCs *in vivo* was delayed in comparison with that of MSCs derived from oro-facial bone [169]. Interestingly, it has also been shown that irradiation stimulates the expansion of the osteoblast compartment in mouse BM, suggested to be due to the proliferation of a currently unidentified osteogenic MSC population in an attempt to repair the BM niche [200]. Taken together, these findings suggest that MSC subpopulations are likely to differentially execute the DDR following genotoxic insult in the BM niche.

## 27.10 Clinical relevance of mesenchymal stromal cells in radiation injury: two sides to the coin

### 27.10.1 Mesenchymal stromal cells and hematopoietic stem cell transplantation

In an allogeneic HSC transplant (HSCT) the recipient receives chemotherapy or chemotherapy and total body irradiation followed by an inoculum of donor cells that contain hematopoietic stem and progenitor cells. The source of the donor HSCs can be BM, mobilized peripheral blood stem cells, or umbilical cord blood stem cells. These cells subsequently reconstitute the hematopoietic and immune systems of the recipient. Such transplants can be genotypically matched for the antigens of the major histocompatibility complex (MHC) if a sibling is used as the donor, or phenotypically MHC-matched if a living unrelated volunteer donor is used. Additionally, an MHC antigen-mismatched HSCT can be used if an MHC-matched donor is unavailable. Even larger degrees of MHC disparity between HSC donor and HSC recipient can be tolerated if umbilical cord blood stem cells are used.

For the past 50 years, allogeneic HSCT has remained the most effective treatment for a number of lymphoid and myeloid malignancies. HSCT can also be used as treatment for some genetic disorders, including severe combined immunodeficiency disease, sickle cell anemia, and thalassemia major. Prior to transplantation, as already indicated, the recipient must undergo treatment known as a conditioning or preparative regimen, which consists of chemotherapy or chemotherapy and radiotherapy [201]. This treatment is required to eliminate any malignant cells, as well as any residual recipient immune cells that could mount an immune response to donor cells, leading to allograft rejection and BM failure. Total body irradiation is frequently used prior to allogeneic HSCT because it covers all sites of hematopoiesis and lymphopoiesis, as well as all sites in the body that harbor malignant cells [201,202]. However, there remains a 30% mortality rate from the procedure for those receiving myeloablative chemotherapy or chemoradiation followed by an allogeneic HSCT for advanced hematologic malignancy. Thus, new strategies focused on reducing complications associated with allogeneic HSCT are required to improve clinical outcomes [201,203–207].

The critical role of MSCs as an instructive support system for hematopoiesis suggests that MSCs may be of therapeutic benefit in allogeneic HSCT. It is well established that irradiated MSCs can support hematopoiesis long term *in vitro*. In addition, conditional depletion of MSC subtypes within mouse BM *in vivo* leads to impaired HSC maintenance and function [1,27–29,208]. Therefore, host MSCs within the BM are likely to contribute to hematopoietic engraftment and reconstitution following HSCT. However, high doses of total body irradiation (10 Gy or above) are known to cause damage to the BM microenvironment, particularly to the perivascular niche [31,209]. In addition, IR-induced damage to the BM vasculature can impede MSC recovery in mouse femurs [209]. Therefore, while irradiated MSCs can support hematopoiesis, loss of the overall BM architecture due to the radiosensitivy of other non-hematopoietic cell types within the niche may indirectly impede hematopoietic recovery following HSCT. Therefore, improving our understanding of the radiation response of the BM niche could enable preconditioning regimens to be improved so that treatments would adequately deplete host hematopoietic cells while maintaining MSCs and other supportive BM niche cells in the BM. In this way it may be possible to improve HSC engraftment and reconstitution without increasing the risk of allograft rejection, thereby potentially increasing the success rates of allogeneic HSC transplantation.

One of the most adverse complications of allogeneic HSC transplantation is graft-versus-host disease (GVHD), which occurs due to the recognition of host tissues as foreign by transplanted donor T cells. Donor T cells mount an immune response against host tissues even in MHC genotypically matched recipients because of the presence of non-MHC histocompatibility antigens present on the cells of the recipient but not on those of the donor. Acute GVHD (defined as occurring during the first 100 days posttransplant) is characterized by excessive production of proinflammatory cytokines and the activation of different immune cells against recipient (or host) tissues, leading to tissue damage in the recipient, which can be fatal and which occurs in spite of prophylactic immune suppressive treatment given to the recipient to minimize the activity of the donor T cells. Acute GVHD mainly affects the recipient's skin, gastrointestinal tract, and liver. Additionally, more than 50% of patients who survive beyond 100 days after allogeneic HSCT require

immunosuppressive treatment for 2 years or more because of chronic (late) GVHD [203,210].

As well as regulating hematopoiesis, MSCs are potent modulators of innate and adaptive immune cell function (see Chapters 33 and 34). MSCs home to sites of inflammation (see Chapters 22 and 23), where they function to suppress the immune response through various mechanisms, including direct cell–cell contact and the production of immunomodulatory factors.

MSCs suppress the functions of various immune cell types implicated in allograft rejection and in GVHD, particularly T cells, DCs, and natural killer (NK) cells. DCs display antigenic peptides to T lymphocytes, promoting T cell activation in an adaptive immune system response [211]. NK cells are cytotoxic lymphocytes that produce proinflammatory cytokines such as tumor necrosis factor-α and interferon-γ during an innate immune system response, which stimulates the recruitment and activation of other immune cells, thus further driving inflammation [211,212]. MSCs inhibit NK cell proliferation, cytotoxic activity, and secretion of proinflammatory cytokines by cell-mediated interactions and via soluble factors, including indoleamine 2,3-dioxygenase and prostaglandin $E_2$ [213]. MSCs also inhibit (1) the differentiation of myeloid precursors into DCs and (2) interactions between DCs and lymphocytes, thus again suppressing the immune response [212–214]. T cells, which are responsible for adaptive cell-mediated immunity, interact directly with DCs, which express histocompatability or other peptide antigens on their surface. This interaction results in T cells mediating antigen-specific cytotoxicity and producing cytokines that activate other immune cell types, leading to damage of histoincompatible cells (the GVHD response), virally infected cells, or residual malignant cells (the graft-versus-leukemia effect) [211]. MSCs suppress the proliferation of mature $CD4^+$ helper and $CD8^+$ cytotoxic T lymphocytes by secreting paracrine factors, including indoleamine 2,3-dioxygenase, prostaglandin $E_2$, nitric oxide, and transforming growth factor-β. MSCs also skew the maturation of naive $CD4^+$ T lymphocytes toward immunosuppressive/regulatory T cell subsets, including T helper 2 cells and regulatory T cells [214–217].

Co-administration of MSCs with donor HSCs has been demonstrated to improve hematopoietic engraftment and reconstitution in irradiated mice and in human allogeneic HSCT recipients [217–222]. Interestingly, MSCs have been shown to home toward radiation-injured non-hematopoietic tissues in which they likely promote wound healing via the production of anti-inflammatory cytokines (such as interleukin-10), growth factors (such as fibroblast growth factor and hepatocyte growth factor), and proangiogenic factors (such as vascular endothelial growth factor) [213,221–224]. Improved hematopoietic recovery observed with MSC co-administration of MSCs and HSCs may be due to the capacity of MSCs to (1) suppress inflammation during allogeneic immune responses such as GVHD and (2) to promote wound healing and tissue regeneration occurring as a result of the pretransplant chemotherapy or chemoradiation. MSC administration is now emerging as a possible therapeutic option for both acute and chronic GVHD. Several phase I and II clinical trials have suggested that MSC administration prevents allograft rejection, improves hematopoietic engraftment, and reduces the severity of GVHD [225–230] and see Chapter 57.

Whether transplanted MSCs directly support and instruct donor hematopoietic cells within the irradiated BM niche is largely unknown. Currently, the study of MSCs in transplantation settings remains technically challenging. When injected intravenously into mice, MSC engraftment in the BM is often poor due to their lodgment in other organs, particularly the lungs. To what extent donor MSCs (1) engraft in the irradiated host BM microenvironment and (2) whether donor MSCs improve hematopoietic reconstitution in comparison with irradiated host MSCs remain to be elucidated.

While several small-scale early phase clinical trials have shown encouraging results, and while MSC administration appears safe, large double-blind, randomized, placebo-controlled phase III clinical trials need to mature before the utility of MSCs in the setting of allogeneic HSCT can be determined.

### 27.10.2 Mesenchymal stromal cells and the tumor microenvironment

The capacity of MSCs to (1) home to sites of injury and inflammation, (2) to repair damaged tissues, and (3) to modulate immune responses is likely to be beneficial for tissue recovery following IR. However, these characteristics could equally prove to be detrimental in the context of cancer therapy. Solid tumors consist of neoplastic cells that are supported by a heterogeneous population of stromal and immune cells, comprising the tumor microenvironment. Tumor formation causes changes in the tissue environment that mimic those of a chronic wound site with tissue injury and inflammation. Inflammatory signals (such as interleukin-6 and interferon-γ) and chemokines (including CXCL8, monocyte chemoattractant protein 1, and CXCL12) that are produced at the tumor site stimulate MSC recruitment into the tumor microenvironment [231–235]. Once integrated in a tumor, MSCs are transformed into cancer-associated fibroblasts (CAFs) through currently unknown mechanisms [236,237]. CAFs promote tumorigenesis by (1) inhibiting apoptosis of cancer cells, (2) secreting growth factors that stimulate cancer cell growth, (3) secreting angiogenic factors and differentiating into pericytes, contributing to the tumor vasculature, (4) suppressing the activity of antitumor immune cells that normally act to eliminate cancerous cells, and (5) producing ECM molecules such as collagen and periostin and promigratory chemokines such CCL5 that promote tissue invasion and metastasis [234–239]. Since MSCs are radioresistant, MSC-derived CAFs within the tumor microenvironment may not be ablated by conventional radiotherapy. Consequently, these CAFs may continue to support the growth of therapy-resistant cancer cells and cancer stem cells, preventing tumor elimination and subsequently leading to tumor recurrence and spread.

MSCs within the BM niche are implicated in promoting the development and treatment resistance of hematological malignancies, particularly leukemias. MSCs produce cell–cell adhesion molecules such as vascular cell adhesion molecule-1 and fibronectin and factors such as CXCL12 and SCF that stimulate the homing and engraftment of leukemic cells. Leukemic cells establish malignant niches in which leukemic stem cells enter a quiescent state and in which they are highly resistant to antileukemia treatments [240–247]. MSCs also contribute to the treatment resistance of leukemic cells by suppressing apoptosis. For example, the interaction of MSC-derived CXCL12 with CXCR4 expressed on leukemic cells can (1) suppress chemotherapy-induced caspase-3 activation and (2) induce increased expression of antiapoptotic proteins such as Bcl-2, Bcl-xl, and myeloid cell leukemia 1 in leukemic cells [248–250].

Solid tumors often contain an abnormal, disorganized vascular network, resulting in heterogeneously distributed hypoxic regions within the tumor mass. Intratumor hypoxia is an adverse prognostic factor in cancer associated with increased malignancy, therapeutic resistance, and metastasis. The increased radioresistance of hypoxic cancer cells is thought to be mediated by two factors: (1) a reduction in IR-induced reactive oxygen species production, which causes chemical alterations in DNA that contribute to the generation of DNA damage; and (2) the activation of signaling cascades, mainly regulated by HIF-1α and HIF-2α, which lead to biological alterations in hypoxic cancer cells that promote resistance to treatment and genomic instability [45,191]. Hypoxia has been shown to enhance MSC radioresistance, particularly by upregulating DNA DSB repair in an HIF-1α-dependent manner [166]. These findings suggest that hypoxia may also contribute to tumorigenesis by altering the radioresistance of the tumor stroma, including MSC-derived CAFs. Similar to the tumor microenvironment, the BM niche is also relatively hypoxic. Hypoxia has been shown to improve the ability of MSCs to support leukemic cells in the BM and to promote leukemic stem cell survival and therapeutic resistance [251,252]. Thus, the enhanced resistance of hypoxic MSCs to DNA-damaging agents, including IR, may also promote the survival of leukemic cells in the BM, potentially contributing to clonal outgrowth of treatment-refractory cells and recurrence of disease.

Although MSCs play important roles in supporting tumorigenesis and leukemogenesis, there is little evidence to suggest that MSCs themselves undergo malignant transformation [253]. Currently, malignant transformation of MSCs has only been directly implicated in osteogenic sarcoma [254]. This rare form of sarcoma mainly occurs in children and young adults and exhibits osteoblastic differentiation and the production of osteoid tissue with a high density of malignant cells within bone. Mutations in the cell cycle regulator genes *p53* and *Rb* are frequently found in osteosarcoma cells and accelerate tumor development in mouse models [254–256]. Ewing's sarcoma is another rare form of bone tumor thought to be derived from neuroectodermal cells, although they are sometimes classified as mesodermal in origin [257]. These findings suggest that the maintenance of an intact DDR in MSCs is likely to be important for regulating MSC growth, differentiation, and function *in vivo*.

In conclusion, the radioresistance of MSCs has several significant implications for the clinic. It is evident that MSC radioresistance can be either beneficial or harmful, and this should be taken into account when designing therapeutic regimens using MSCs. MSCs may have beneficial effects in treating GVHD and improving allogeneic HSCT outcomes. Continued investigation into MSC radioresistance will have an impact on our understanding of how MSCs (1) restore hematopoiesis, (2) modulate the immune system, and (3) sustain cancer development in response to DNA-damaging agents such as IR. It is thus possible that they help develop more effective therapies for cancer.

## References


1 L. Ding, T.L. Saunders, G. Enikolopov, and S.L. Morrison. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 2012;**481**:457–462.

2 P. Eliasson and J.I. Jönsson. The hematopoietic stem cell niche: low in oxygen but a nice place to be. *J Cell Physiol* 2010;**222**:17–22.

3 Z. Li and L. Li. Understanding hematopoietic stem-cell microenvironments. *Trends Biochem Sci* 2006;**31**:589–595.

4 C.L. Celso and D.T. Scadden. The haematopoietic stem cell niche at a glance. *J Cell Sci* 2011;**124**:3529–3535.

5 A. Ehninger and A. Trumpp. The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. *J Exp Med* 2011;**208**:421–428.

6 L.D. Wang and A.J. Wagers. Dynamic niches in the origination and differentiation of haematopoietic stem cells. *Nat Rev Mol Cell Biol* 2011;**12**:643–655.

7 A.J. Friedenstein, R.K. Chailakhjan, and K.S. Lalykina. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**:393–403.

8 A.J. Friedenstein, R.K. Chailakhjan, N.V. Latsinik, *et al.* Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. *Transplantation* 1974;**17**:331–340.

9 A.J. Friedenstein, I.I. Piatetzky-Shakiro, and K.V. Petrakova. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;**16**:381–390.

10 A.E. Grigoriadis, N.J. Heersche, and J.E. Aubin. Differentiation of muscle, fat, cartilage and bone from progenitor cells present in a bone derived clonal cell population: effect of dexamethasone. *J Cell Physiol* 1988;**106**:2139–2151.

11 S.A. Kuznetsov, P.H. Krebsbach, K. Satomura, *et al.* Single-colony derived strains of human marrow stromal fibroblasts form bone after transplantation *in vivo*. *J Bone Miner Res* 1997;**12**:1335–1347.

12 M.F. Pittenger, A.M. Mackay, S.C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.

13 S. Morikawa, Y. Mabuchi, Y. Kubota, *et al.* Prospective identification, isolation, and systemic transplantation of multipotent mesenchymal stem cells in murine bone marrow. *J Exp Med* 2009;**206**:2483–2496.

14 J.A. Kode, S. Mukherjee, M.V. Joglekar, and A.A. Hardikar. Mesenchymal stem cells: immunobiology and role in immunomodulation and tissue regeneration. *Cytotherapy* 2009;**11**:377–391.

15 J. Tolar, K. Le Blanc, A. Keating, and B.R. Blazar. Concise review: hitting the right sport with mesenchymal stromal cells. *Stem Cells* 2010;**28**:1446–1455.

16 J.E. Till and E.A. McCulloch. A direct measurement of the radiation sensitivity of mouse bone marrow cells. *Radiat Res* 1961;**14**:213–222.

17 M.C. Magli, N.N. Iscove, and N. Odartchenko. Transient nature of early haematopoietic spleen colonies. *Nature* 1982;**295**:527–529.

18 T.M. Dexter, T.D. Allen, and L.G. Lajtha. Conditions controlling the proliferation of haemopoietic stem cells *in vitro*. *J Cell Physiol* 1977;**91**:335–344.

19 C.A. Whitlock and O.N. Witte. Long-term culture of B lymphocytes and their precursors from murine bone marrow. *Proc Natl Acad Sci U S A* 1982;**79**:3608–3612.

20 P.J. Quesenberry. Stromal cells in long-term bone marrow cultures. In: M. Tavassoli (ed.), *Handbook of the Hematopoietic Microenvironment*. Humana Press, 1990; pp. 253–285.

21 D. Park, J.A. Spencer, B.I. Koh, *et al.* Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* 2012;**10**:259–272.

22 L.M. Calvi, G.B. Adams, K.W. Weibrecht, *et al.* Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* 2003;**425**:841–846.

23 J. Zhang, C. Niu, L. Ye, *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003;**425**:836–841.

24 R.S. Taichman. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem cell niche. *Blood* 2005;**105**:2631–2639.

25 A. Greenbaum, Y.M. Hsu, R.B. Day, *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* 2013;**495**:227–230.

26 Y. Omatsu, T. Sugiyama, H. Kohara, *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity* 2010;**33**:387–399.

27 E.W. Roberts, A. Deonarine, O.J. Jones, *et al.* Depletion of stromal cells expressing fibroblast activation protein-α from skeletal muscle and bone marrow results in cachexia and anemia. *J Exp Med* 2013;**210**:1137–1151.

28 E. Tran, D. Chinnasamy, Z. Yu, *et al.* Immune targeting of fibroblast activation protein triggers recognition of multipotent bone marrow stromal cells and cachexia. *J Exp Med* 2013;**210**:1125–1135.

29 S. Méndez-Ferrer, T.V. Michinura, F. Ferraro, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

30 A. Moyheldin, T. Garzón-Muvdi, and A. Quiñones-Hinojosa. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell* 2010;**7**:150–161.

31 J.A. Spencer, F. Ferraro, E. Roussakis, *et al.* Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* 2014;**508**:269–273.

32 U. Lendahl, K.L. Lee, H. Yang, and L. Poellinger. Generating specificity and diversity in the transcriptional response to hypoxia. *Nat Rev Genet* 2009;**10**:821–832.

33 G.L. Semenza. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. *J Appl Physiol* 2000;**88**:1474–1480.

34 Q. Ke and M. Costa. Hypoxia-inducible factor-1 (HIF-1). *Mol Pharmacol* 2006;**70**:1469–1480.

35 K. Ruan, G. Song, and G. Ouyang. Role of hypoxia in the hallmarks of human cancer. *J Cell Biochem* 2009;**107**:1053–1062.

36 A.V. Guitart, M. Hammoud, P.D. Sbarba, *et al.* Slow cycling/quiescence balance of hematopoietic stem cells is related to physiological gradient of oxygen. *Exp Hematol* 2012;**38**:847–851.

37 I.G. Winkler, V. Barbier, R. Wadley, *et al.* Positioning of bone marrow hematopoietic and stromal cells relative to blood flow *in vivo*: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. *Blood* 2010;**116**:375–385.

38 Y. Jin, T. Kato, N. Furu, *et al.* Mesenchymal stem cells cultured under hypoxia escape from senescence via down-regulation of p16 and extracellular signal regulated kinase. *Biochem Biophys Res Commun* 2010;**391**:1471–1476.

39 C.C. Tsai, P.F. Su, Y.F. Huang, *et al.* Oct4 and Nanog directly regulate Dnmt1 to maintain self-renewal and undifferentiated state in mesenchymal stem cells. *Mol Cell* 2012;**47**:169–182.

40 C.C. Tsai, T.L. Yew, D.C. Yang, *et al.* Benefits of hypoxic culture on bone marrow multipotent stromal cells. *Am J Blood Res* 2012;**2**:148–159.

41 L. Liu, J. Gao, Y. Yuan, *et al.* Hypoxia preconditioned human adipose derived mesenchymal stem cells enhance angiogenic potential via secretion of increased VEGF and bFGF. *Cell Biol Int* 2013;**37**:551–560.

42 S. Prado-Lòpez, M.M. Duffy, C. Baustian, *et al.* The influence of hypoxia on the differentiation capacities and immunosuppressive properties of clonal mouse mesenchymal stromal cell lines. *Immunol Cell Biol* 2014;**92**:612–623.

43 T.M. Fliedner, D. Graessle, C. Paulsen, and K. Reimers. Structure and function of bone marrow hemopoiesis: mechanisms of response to ionizing radiation exposure. *Cancer Biother Radiopharm* 2002;**17**:405–426.

44 N. Dainiak, J.K. Waselenko, J.O. Armitage, *et al.* The hematologist and radiation casualties. *Hematol Am Soc Hematol Educ Program* 2003;**2003**:473–496.

45 E.J. Hall and A.J. Giaccia. *Radiobiology for the Radiologist*, 6th edn. Lippincott Williams and Wilkins, 2006.

46 A.J. Friedenstein, J.F. Gorskaja, and N.N. Kulagina. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;**4**:267–274.

47 J. Laver, S.C. Jhanwar, R.J. O'Reilly, and H. Castro-Malaspina. Host origin of the human hematopoietic microenvironment following allogeneic bone marrow transplantation. *Blood* 1987;**70**:1966–1968.

48 P.J. Simmons, D. Przepiorka, E.D. Thomas, and B. Torok-Storb. Host origin of marrow stromal cells following allogeneic bone marrow transplantation. *Nature* 1987;**328**:429–432.

49 K. Arematsu and Y. Nakahori. Recipient origin of bone marrow-derived fibroblastic stromal cells during all periods following bone marrow transplantation in humans. *Br J Haematol* 1991;**79**:359–365.

50 K. Rieger, O. Marinets, T. Fietz, *et al.* Mesenchymal stem cells remain of host origin even a long time after allogeneic peripheral blood stem cell or bone marrow transplantation. *Exp. Hematol.* 2005;**33**:605–611.

51 K. Bartsch, H. Al-Ali, A. Reinhardt, *et al.* Mesenchymal stem cells remain host-derived independent of the source of the stem-cell

graft and conditioning regimen used. *Transplantation* 2009;**87**: 217–221.
52 M.B. Kastan and J. Bartek. Cell cycle checkpoints and cancer. *Nature* 2004;**432**:316–323.
53 A. Ciccia and S.J. Elledge. The DNA damage response: making it safe to play with knives. *Mol Cell* 2010;**40**:179–204.
54 J.F. Ward. DNA damage produced by ionizing radiation in mammalian cells: identities, mechanisms of formation and reparability. *Prog Nucleic Acid Res Mol Biol* 1988;**35**:95–125.
55 J.H. Houtgraff, J. Versmissen, and W.J. van der Giessen. A concise review of DNA damage checkpoints and repair in mammalian cells. *Cardiovasc Revasc Med* 2006;**7**:165–172.
56 N.F. Lowndes and J.R. Murguia. Sensing and responding to DNA damage. *Curr Opin Genet Dev* 2000;**10**:17–25.
57 C. Redon, D.R. Pilch, E.P. Rogakou, *et al.* Yeast histone 2A serine 129 is essential for the efficient repair of checkpoint-blind DNA damage. *EMBO Rep* 2003;**4**:678–684.
58 D. Branzei and M. Foiani. The DNA damage response during DNA replication. *Curr Opin Cell Biol* 2005;**17**:568–575.
59 R.S. Cha and N. Kleckner. ATR homolog Mec1 promotes fork progression, thus averting breaks in slow replication zones. *Science* 2002;**297**:602–606.
60 S. Lambert and A.M. Carr. Checkpoint responses to replication barriers. *Biochimie* 2005;**87**:591–602.
61 Y. Shiloh and Y. Ziv. The ATM protein kinase: regulating the cellular response to genotoxic stress, and more. *Nat Rev Mol Cell Biol* 2013;**14**:197–210.
62 K. Rothkamm, I. Krüger, L.H. Thompson, and M. Löbrich. Pathways of DNA double-strand break repair during the mammalian cell cycle. *Mol Cell Biol* 2003;**23**:5706–5715.
63 K.A. Kimprich and D. Cortez. ATR: an essential regulator of genome integrity. *Nat Rev Mol Cell Biol* 2008;**9**:616–627.
64 Y. Shiloh. ATM and related protein kinases: safeguarding genome integrity. *Nat Rev Cancer* 2003;**3**:155–168.
65 P.J. Hurley and F. Bunz. ATM and ATR: components of an integrated circuit. *Cell Cycle* 2007;**6**:414–417.
66 C. Beck, I. Robert, B. Reina-San-Martin, *et al.* Poly(ADP-ribose) polymerases in double-strand break repair: focus on PARP1, PARP2 and PARP3. *Exp Cell Res* 2014;**329**:18–25.
67 C. Boehler, L.R. Gauthier, O. Mortusewicz, *et al.* Poly(ADP-ribose) polymerase 3 (PARP3), a newcomer in cellular response to DNA damage and mitotic progression. *Proc Natl Acad Sci U S A* 2011;**108**:2783–2788.
68 J. Lukas, C. Lukas, and J. Bartek. More than just a focus: the chromatin response to DNA damage and its role in genome integrity maintenance. *Nat Cell Biol* 2011;**13**:1161–1169.
69 A. Rupnik, N.F. Lowndes, and M. Grenon. MRN and the race to the break. *Chromosoma* 2010;**119**:115–135.
70 G.J. Williams, S.P. Lees-Miller, and J.A. Tainer. Mre11–Rad50–Nbs1 conformations and the control of sensing, signaling, and effector responses at DNA double-strand breaks. *DNA Repair (Amst)* 2010;**9**:1299–1306.
71 B.J. Lamarche, N.I. Orazio, and M.D. Weitzman. The MRN complex in double-strand repair and telomere maintenance. *FEBS Lett* 2010;**584**:3682–3695.
72 K.P. Hopfner, A. Karcher, L. Craig, *et al.* Structural biochemistry and interaction architecture of the DNA double-strand break repair Mre11 nuclease and Rad50-ATPase. *Cell* 2001;**105**:473–485.
73 J.H. Lee and T.T. Paull. ATM activation by DNA double-strand breaks through the Mre11–Rad50–Nbs1 complex. *Science* 2005; **308**:551–554.
74 C.J. Bakkenist and M.B. Kastan. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature* 2003;**421**:499–506.
75 Y. Sun, Y. Xu, K. Roy, and B.D. Price. DNA damage-induced acetylation of lysine 3016 of ATM activates ATM kinase activity. *Mol Cell Biol* 2007;**27**:8502–8509.
76 M. Pellegrini, A. Celeste, S. Difilippantonio, *et al.* Autophosphorylation at serine 1987 is dispensable for murine Atm activation *in vivo*. *Nature* 2006;**443**:222–225.
77 A. Gospodinov and Z. Herceg. Chromatin structure in double strand break repair. *DNA Repair (Amst)* 2013;**12**:800–810.
78 M.J. Kruhlak, A. Celeste, and A. Nussenzweig. Spatio-temporal dynamics of chromatin containing DNA breaks. *Cell Cycle* 2006;**5**:1910–1912.
79 G. Smeenk, W.W. Wiegant, J.A. Marteijn, *et al.* Poly(ADP-ribosyl)ation links the chromatin remodeler SMARCA5/SNF2H to RNF168-dependent DNA damage signaling. *J Cell Sci* 2013;**126**:889–903.
80 R. Kumar, N. Horikoshi, M. Singh, *et al.* Chromatin modifications and the DNA damage response to ionizing radiation. *Front Oncol* 2013;**2**:1–9.
81 N.F. Lowndes. The interplay between BRCA1 and 53BP1 influences death, aging, senescence and cancer. *DNA Repair (Amst)* 2010;**9**: 1112–1116.
82 B.D. Price and A.D. D'Andrea. Chromatin remodelling at DNA double-strand breaks. *Cell* 2013;**152**:1344–1353.
83 J.W. Harper and S.J. Elledge. The DNA damage response: ten years after. *Mol. Cell* 2007;**28**:739–745.
84 E.P. Rogakou, D.R. Pilch, A.H. Orr, *et al.* DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem* 1998;**273**:5858–5868.
85 S. Burma, B.P. Chen, M. Murphy, *et al.* ATM phosphorylates histone H2AX in response to DNA double-strand breaks. *J Biol Chem* 2001;**276**:42462–42467.
86 W.M. Bonner, C.E. Redon, J.S. Dickey, *et al.* γH2AX and cancer. *Nat Rev Cancer* 2008;**8**:957–967.
87 O. Fernandez-Capetillo, A. Lee, M. Nussenzweig, and A. Nussenzweig. H2AX: the histone guardian of the genome. *DNA Repair (Amst)* 2004;**3**:959–967.
88 V. Savic, B. Yin, N.L. Maas, *et al.* Formation of dynamic gamma-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent on H2AX densities in chromatin. *Mol Cell* 2009;**34**:298–310.
89 G. Sulli, R. Di Micco, and F. d'Adda di Fagagna. Crosstalk between chromatin state and DNA damage response in cellular senescence and cancer. *Nat Rev Cancer* 2012;**12**:709–720.
90 N.F. Lowndes and G.W. Toh. DNA repair: the importance of phosphorylating histone H2AX. *Curr Biol* 2005;**15**:R99–R102.
91 L. Zou and S.J. Elledge. Sensing DNA damage through ATRIP recognition of RPA–ssDNA complexes. *Science* 2003;**300**:1542–1548.
92 M. Cuadrado, B. Martinez-Pastor, M. Murga, *et al.* ATM regulates ATR chromatin loading in response to DNA double-strand breaks. *J Exp Med* 2006;**203**:297–303.
93 A. Jazayeri, J. Falck, C. Lukas, *et al.* ATM- and cell cycle-dependent regulation of ATR in response to DNA double-strand breaks. *Nat Cell Biol* 2006;**8**:37–45.

94 C. Norbury and P. Nurse. Animal cell cycles and their control. *Annu Rev Biochem* 1992;**61**:441–470.

95 E.D. Israels and L.G. Israels. The cell cycle. *Oncologist* 2000; **5**(6):510–513.

96 E. Zucca and E.A. Nigg. Cell cycle regulation and the function of cancer genes. *Ann Oncol* 1995;**6**:975–978.

97 N.C. Walworth. Cell-cycle checkpoint kinases: checking in on the cell cycle. *Curr Opin Cell Biol* 2006;**12**:697–704.

98 K. Vermuelen, D.R. Van Bockstaele, and Z.N. Berneman. The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif* 2003;**36**:131–149.

99 J. Massagué. $G_1$ cell-cycle control and cancer. *Nature* 2004;**432**: 298–306.

100 P. Fei and W.S. El-Deiry. p53 and radiation responses. *Oncogene* 2003;**22**:5774–5783.

101 A.L. Gartel and S.K. Radhakrishnan. Lost in transcription: p21 repression, mechanisms and consequences. *Cancer Res* 2005;**65**: 3980–3985.

102 D. Deckbar, P.A. Jeggo, and M. Löbrich. Understanding the limitations of radiation-induced cell cycle checkpoints. *Crit Rev Biochem Mol Biol* 2011;**46**:271–283.

103 D. Deckbar, T. Stiff, B. Koch, *et al.* The limitations of the $G_1$–S checkpoint. *Cancer Res* 2010;**70**:4412–4421.

104 J. Bartek and J. Lukas. Mammalian $G_1$- and S-phase checkpoints in response to DNA damage. *Curr Opin Cell Biol* 2001;**13**: 738–747.

105 J. Bartek and J. Lukas. Chk1 and Chk2 kinases in checkpoint control and cancer. *Cancer Cell* 2003;**3**:421–429.

106 N. Mailand, J. Falck, C. Lukas, *et al.* Rapid destruction of Cdc25A in response to DNA damage. *Science* 2000;**288**:1425–1429.

107 R.S. Tibbetts, K.M. Brumbaugh, J.M. Williams, *et al.* A role for ATR in the DNA damage-induced phosphorylation of p53. *Genes Dev* 1999;**13**:152–157.

108 J.K. Eykelenboom, E.C. Harte, L. Canavan, *et al.* ATR activates the S–M checkpoint during unperturbed growth to ensure sufficient replication prior to mitotic onset. *Cell Rep* 2013;**5**:1095–1107.

109 J. Falck, N. Mailand, R.G. Syljuåsen, *et al.* The ATM–Chk2–Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature* 2001;**410**:842–847.

110 J. Bartek, C. Lukas, and J. Lukas. Checking on DNA damage in S phase. *Nat Rev Mol Cell Biol* 2004;**5**:792–803.

111 R. Sherwood, T.S. Takahasi, and P.V. Jallepalli. Sister acts: coordinating DNA replication and cohesion establishment. *Genes Dev* 2010;**24**:2723–2731.

112 C. Bauerschmidt, C. Arrichiello, S. Burdak-Rothkamm, *et al.* Cohesin promotes the repair of ionizing radiation-induced DNA double-strand breaks in replicated chromatin. *Nucleic Acids Res* 2010;**38**:477–487.

113 R. Kitigawa, C.J. Bakkenist, P.J. McKinnon, and M.B. Kasten. Phosphorylation of SMC1 is a critical downstream event in the ATM–NBS1–BRCA1 pathway. *Genes Dev* 2004;**18**:1423–1438.

114 H. Luo, Y. Li, J.J. Mu, *et al.* Regulation of the intra-S phase checkpoint by ionizing radiation-(IR) dependent and IR-independent phosphorylation of SMC3. *J Biol Chem* 2008;**283**: 19176–19183.

115 J. Lukas, C. Lukas, and J. Bartek. Mammalian cell cycle checkpoints: signalling pathways and their organization in space and time. *DNA Repair (Amst)* 2004;**3**:997–1007.

116 R.T. Abraham. Cell cycle checkpoint signaling through the ATM and ATR kinases. *Genes Dev* 2001;**15**:2177–2196.

117 A. Shibata, O. Barton, A.T. Noon, *et al.* Role of ATM and damage response mediator proteins 53BP1 and MDC1 in the maintenance of $G_2$/M checkpoint arrest. *Mol Cell Biol* 2010;**30**:3371–3383.

118 J.M. Daley and P. Sung. 53BP1, BRCA1, and the choice between recombination and end joining at DNA double-strand breaks. *Mol Cell Biol* 2014;**34**:1380.

119 J.R. Chapman, P. Barral, J.B. Vannier, *et al.* RIF1 is essential for 53BP1-dependent nonhomologous end joining and suppression of DNA double-strand break resection. *Mol Cell* 2013;**49**:858–871.

120 T. Iyama and D.M. Wilson III. DNA repair mechanisms in dividing and non-dividing cells. *DNA Repair (Amst)* 2013;**12**:620–636.

121 A. Beucher, J. Birraux, L. Tchouandong, *et al.* ATM and Artemis promote homologous recombination of radiation-induced DNA double-strand breaks in $G_2$. *EMBO J* 2009;**28**:3413–3427.

122 A.J. Hartlerode and R. Scully. Mechanisms of double-strand break repair in somatic mammalian cells. *Biochem J* 2009;**423**: 157–168.

123 P.A. Jeggo and M. Löbrich. Contribution of DNA repair and cell cycle checkpoint arrest to the maintenance of genomic stability. *DNA Repair (Amst)* 2006;**5**:1192–1198.

124 K.P. Hopfner, C.D. Putnam, and J.A. Tainer. DNA double-strand break repair from head to tail. *Curr Opin Struct Biol* 2002;**12**: 115–122.

125 M.R. Lieber, Y. Ma, U. Pannicke, and K. Schwarz. Mechanism and regulation of human non-homologous DNA end-joining. *Nat Rev Mol Cell Biol* 2003;**4**:712–720.

126 M. Zimmermann, F. Lottersberger, S.B. Buonomo, *et al.* 53BP1 regulates DSB repair using Rif1 to control 5′ end resection. *Science* 2013;**339**:700–704.

127 S. Difilippantonio, E. Gapud, N. Wong, *et al.* 53BP1 facilitates long-range DNA end-joining during V(D)J recombination. *Nature* 2008;**456**:529–534.

128 A. Bothmer, D. F. Robbiani, M. Di Virgilio, *et al.* Regulation of DNA end joining, resection, and immunoglobulin class switch recombination by 53BP1. *Mol Cell* 2011;**42**:319–329.

129 I. Rybanska-Spaeder, T.L. Reynolds, J. Chou, *et al.* 53BP1 is limiting for nonhomologous end-joining in ATM-deficient cells subjected to oncogenic stress or radiation. *Mol Cancer Res* 2013;**11**: 1223–1234.

130 X. Li and W.D. Heyer. Homologous recombination in DNA repair and DNA damage tolerance. *Cell Res* 2008;**18**:99–113.

131 O.S. Gildemeister, J.M. Sage, and K.L. Knight. Cellular distribution of Rad51 in response to DNA damage: novel role for Rad51C. *J Biol Chem* 2009;**284**:31945–31952.

132 A.A. Sartori, C. Lukas, J. Coates, *et al.* Human CtIP promotes DNA end resection. *Nature* 2007;**22**:509–514.

133 L.S. Symington, and J. Gautier. Double-strand break end resection and repair pathway choice. *Annu Rev Genet* 2011;**45**:247–271.

134 C. Escribano-Díaz, A. Orthwein, A. Fradet-Turcotte, *et al.* A cell cycle-dependent regulatory circuit composed of 53BP1–RIF1 and BRCA1–CtIP controls DNA repair pathway choice. *Mol Cell* 2013;**49**:872–883.

135 K.A. Bernstein and R. Rothstein. At loose ends: resecting a double-strand break. *Cell* 2009;**137**:807–810.

136 S. Paniel and S. J. Boulton. Double-strand break repair: 53BP1 comes into focus. *Nat Rev Mol Cell Biol* 2013;**15**:7–18.

137 M.H. Yun and K. Hiom. CtIP–BRCA1 modulates the choice of DNA double-strand break repair pathway throughout the cell cycle. *Nature* 2009;**459**:460–464.

138 M. Zimmermann and T. de Lange. 53BP1: pro choice in DNA repair. *Trends Cell Biol* 2014;**24**:108–117.

139 S. Nowsheen and E.S. Yang. The intersection between DNA damage response and cell death pathways. *Exp Oncol* 2012;**34**:243–254.

140 O. Surova and B. Zhivotovsky. Various modes of cell death induced by DNA damage. *Oncogene* 2013;**32**:3789–3797.

141 M.S. Ola, M. Nawaz, and H. Ahsan. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Mol Cell Biochem* 2011;**351**:41–58.

142 W.P. Roos and B. Kaina. DNA damage-induced cell death by apoptosis. *Trends Mol Med* 2006;**12**:440–450.

143 B. Zhivotovsky and G. Kroemer. Apoptosis and genomic instability. *Nat Rev Mol Cell Biol* 2004;**5**:752–762.

144 M.O. Hengartner. The biochemistry of apoptosis. *Nature* 2000;**407**: 770–776.

145 A. Strasser, S. Cory, and J.M. Adams. Deciphering the rules of programmed cell death to improve therapy of cancer and other diseases. *EMBO J* 2011;**30**:3667–3683.

146 J.M. Adams and S. Cory. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007;**26**:1324–37.

147 S.N. Willis and J.M. Adams. Life in the balance: how BH3-only proteins induce apoptosis. *Curr Opin Cell Biol* 2005;**17**:617–625.

148 P. Bouillet, D. Metcalf, D.C.S Huang, *et al.* Proapoptotic Bcl-2 relative Bim is required for certain apoptotic responses, leukocyte homeostasis and to preclude autoimmunity. *Science* 1999;**286**: 1735–1738.

149 M. Erlacher, E.M. Michalak, P.N. Kelly, *et al.* BH3-only proteins Puma and Bim are rate-limiting for γ-radiation- and glucocorticoid-induced apoptosis of lymphoid cells *in vivo*. *Blood* 2005;**106**:4131–4138.

150 F.J. Oliver, J. Menissier-de Murcia, and G. de Murcia. Poly (ADP-ribose) polymerase in the cellular response to DNA damage, apoptosis and disease. *Am J Hum Genet* 1999;**64**:1282–1288.

151 J. Campisi and F. d'Adda di Fagagna. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007;**8**:729–740.

152 F. d'Adda di Fagagna. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer* 2008;**8**:512–522.

153 R. Salama, M. Sadaie, M. Hoare, and M. Narita. Cellular senescence and its effector programs. *Genes Dev* 2014;**28**:99–114.

154 H. Rayess, M. B. Wang, and E.S. Srivatsan. Cellular senescence and tumor suppressor gene p16. *Int J Cancer* 2012;**130**:1715–1725.

155 E.I. Meijne, R.J. van der Wingen-van Groenewegen, R.E. Ploemacher, *et al.* The effects of X-irradiation on hematopoietic stem cell compartments in the mouse. *Exp Hematol* 1991;**19**:617–623.

156 T.M. Filion, M. Qiao, P.N. Ghule, *et al.* Survival responses of human embryonic stem cells to DNA damage. *J Cell Physiol* 2009;**220**: 586–592.

157 G. Harfouche and M.T. Martin. Response of normal stem cells to ionizing radiation: a balance between homeostasis and genomic stability. *Mutat Res* 2010;**704**:167–174.

158 P.A. Sotiropoulou, A. Candi, G. Mascré, *et al.* Bcl-2 and accelerated DNA repair mediates resistance of hair follicle bulge stem cells to DNA-damage-induced cell death. *Nat Cell Biol* 2010;**12**:572–582.

159 R. Tibeiro, A. Marconia, C. Fila, *et al.* Keratinocytes enriched for stem cells are protected from anoikis via an integrin signaling pathway in a Bcl-2 dependent manner. *FEBS Lett* 2002;**524**: 139–144.

160 M. Milyavsky, O.I. Gan, M. Trottier, *et al.* A distinctive DNA damage response in human hematopoietic stem cells reveals an apoptosis-independent role for p53 in self-renewal. *Cell Stem Cell* 2010;**7**:186–197.

161 K. Inomata, A. Takahiro, N.T. Binh, *et al.* Genotoxic stress abrogates renewal of melanocyte stem cells by triggering their differentiation. *Cell* 2009;**137**:1088–1099.

162 J. Wang, Q. Sun, Y. Morita, *et al.* A differentiation checkpoint limits hematopoietic stem cell self-renewal in response to DNA damage. *Cell* 2012;**148**:1001–1114.

163 M.-F. Chen, C.-T. Lin, W.-C. Chen, *et al.* The sensitivity of human mesenchymal stem cells to ionizing radiation. *Int J Radiat Oncol Biol Phys* 2006;**66**:244–253.

164 Á.M. Prendergast, S. Cruet-Hennequart, G. Shaw, *et al.* Activation of DNA damage response pathways in human mesenchymal stem cells exposed to cisplatin or γ-irradiation. *Cell Cycle* 2011;**10**: 3768–3777.

165 T. Sugrue, J.A.L. Brown, N.F. Lowndes, and R. Ceredig. Multiple facets of the DNA damage response contribute to the radioresistance of mouse mesenchymal stromal cell lines. *Stem Cells* 2013;**31**:137–145.

166 T. Sugrue, N.F. Lowndes, and R. Ceredig. Hypoxia enhances the radioresistance of mouse mesenchymal stromal cells. *Stem Cells* 2014;**32**:2188–2200.

167 T. Stiff, M. O'Driscoll, N. Rief, *et al.* ATM and DNA-PK function redundantly to phosphorylate H2AX after exposure to ionizing radiation. *Cancer Res* 2004;**64**:2390–2396.

168 H. Wang, M. Wang, H. Wang H, *et al.* Complex H2AX phosphorylation patterns by multiple kinases including ATM and DNA-PK in human cells exposed to ionising radiation and treated with kinase inhibitors. *J Cell Physiol* 2005;**202**:492–502.

169 M. Damek-Poprawa, D. Stefanik, L.M. Levin, and O. Akintoye. Human bone marrow stromal cells display variable anatomic site-dependent response and recovery from irradiation. *Arch Oral Biol* 2010;**55**:358–364.

170 O. Momcilovic, L. Knobloch, J. Fornsalgio, *et al.* DNA damage response in human induced pluripotent stem cells and embryonic stem cells. *PLoS ONE* 2010;**5**:e13410.

171 N.H. Nicolay, E. Sommer, R. Lopez, *et al.* Mesenchymal stem cells retain their defining stem cell characteristics after exposure to ionizing radiation. *Int J Radiat Oncol Biol Phys* 2013;**87**:1171–1178.

172 J. Domen, K.L. Gandy, and I.L. Weissman. Systemic over-expression of BCL-2 in the hematopoietic system protects transgenic mice from the consequences of lethal irradiation. *Blood* 1998;**91**:2272–2282.

173 S. Ogilvy, D. Metcalf, C.G. Print, *et al.* Constitutive bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc Natl Acad Sci U S A* 1999;**96**:14943–14948.

174 K. Ito, A. Hirao, F. Arai, *et al.* Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells. *Nature* 2004;**431**:997–1002.

175 S.M. Chambers, C.A. Shaw, C. Gatza, *et al.* Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol* 2007;**5**:e201.

176 J. Kenyon and S.L. Gerson. The role of DNA damage repair in aging of adult stem cells. *Nucleic Acids Res* 2007;**35**:7557–7565.

177 T. Sperka, J. Wang, and K.I. Rudolph. DNA damage checkpoints in stem cells, ageing and cancer. *Nat Rev Mol Cell Biol* 2012;**13**: 579–580.

178 G. Luo, M.S. Yao, C.F. Bender, *et al.* Disruption of mRad50 causes embryonic stem cell lethality, abnormal embryonic development, and sensitivity to ionizing radiation. *Proc Natl Acad Sci U S A* 1999;**96**:7376–7381.

179 T. Cheng, N. Rodrigues, H. Shen, *et al.* Hematopoietic stem cell quiescence maintained by p21cip1/waf1. *Science* 2000;**287**: 1804–1808.

180 A. Nijnik, L. Woodbine, C. Marchetti, *et al.* DNA repair is limiting for haematopoietic stem cells during ageing. *Nature* 2007;**447**: 686–691.

181 Z. Ju, H. Jiang, M. Jaworski, *et al.* Telomere dysfunction induces environmental alterations limiting hematopoietic stem cell function and engraftment. *Nat Med* 2007;**13**:742–747.

182 C.R. Walkley, J.M. Shea, N.A. Sims, *et al.* Rb regulates interactions between hematopoietic stem cells and their bone marrow microenviroment. *Cell* 2007;**129**:1081–1095.

183 M.H.G.P. Raajimakers, S. Mukherjee, S. Guo, *et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 2010;**464**:852–857.

184 A. Hishiya, M. Ito, H. Aburatani, *et al.* Ataxia telangiectasia mutated (*Atm*) knockout mice as a model of osteopenia due to impaired bone formation. *Bone* 2005;**37**:497–503.

185 M. O'Driscoll and P.A. Jeggo. The role of double-strand break repair – insights from human genetics. *Nat Rev Genet* 2006;**7**:45–54.

186 Y. Ruzankina, C. Pinzon-Guzman, A. Asare, *et al.* Deletion of the developmentally essential gene ATR in adult mice leads to age-related phenotypes and stem cell loss. *Cell Stem Cell* 2007;**1**: 113–126.

187 A. Wilson and A. Trumpp Bone-marrow hematopoietic-stem-cell niches. *Nat Rev Immunol* 2006;**6**:93–106.

188 A. Unruh, A. Ressel, H.G. Mohamed, *et al.* The hypoxia-inducible factor-1α is a negative factor for tumour therapy. *Oncogene* 2003;**22**:3213–3220.

189 P. Vaupel. Tumour microenvironmental physiology and its implications for radiation oncology. *Semin Radiat Oncol* 2004;**14**: 198–206.

190 J.A. Bertout, S.A. Patel, and M.C. Simon. The impact of $O_2$ availability on human cancer. *Nat Rev Cancer* 2008;**8**:967–975.

191 R.G. Bristow and R.P. Hill. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer* 2008;**8**:180–192.

192 N. Rohwer, C. Zasada, S. Kempa, and T. Cramer. The growing complexity of HIF-1α's role in tumorigenesis: DNA repair and beyond. *Oncogene* 2013;**32**:3569–3576.

193 E.M. Hammond, M.J. Dorie, and A.J. Giaccia. ATR/ATM targets are phosphorylated by ATR in response to hypoxia and ATM in response to reoxygenation. *J Biol Chem* 2003;**278**:12207–12213.

194 S.L. Gibson, R.S. Bindra, and P.M. Glazer. Hypoxia-induced phosphorylation of Chk2 in an ataxia telangiectasia mutated-dependent manner. *Cancer Res* 2005;**65**:10734–10741.

195 S.L. Gibson, R.S. Bindra, and P.M. Glazer. CHK2-dependent phosphorylation of BRCA1 in hypoxia. *Radiat Res* 2006;**166**: 646–651.

196 F. Bouquet, M. Ousset, D. Biard, *et al.* A DNA-dependent stress response involving DNA-PK occurs in hypoxic cells and contributes to cellular adaptation to hypoxia. *J Cell Sci* 2011;**124**:1943–1951.

197 S. Wrann, M.R. Kaufmann, R. Wirthner R, *et al.* HIF mediated and DNA damage independent histone H2AX phosphorylation in chronic hypoxia. *Biol Chem* 2013;**394**:519–528.

198 I. Moen and L.E.B. Stuhr. Hyperbaric oxygen therapy and cancer – a review. *Targ Oncol* 2012;**7**:233–232.

199 C.L. Carbonneau, G. Despars, S. Rojas-Sutterlin, *et al.* Ionizing radiation-induced expression of INK4a/ARF in murine bone marrow-derived stromal cell populations interferes with bone marrow homeostasis. *Blood* 2012;**119**:717–726.

200 M. Dominici, V. Rasini, R. Bussolari, *et al.* Restoration and reversible expansion of the osteoblastic hematopoietic stem cell niche after marrow radioablation. *Blood* 2009;**114**:2333–2343.

201 E.A. Copelan. Hematopoietic stem-cell transplantation. *N Engl J Med* 2006;**354**:1813–1826.

202 P. Mauch, L. Constine, J. Greenberger, *et al.* Hematopoietic stem cell compartment: acute and late effects of radiation therapy and chemotherapy. *Int J Radiat Oncol Biol* 1995;**31**: 1319–1339.

203 E.J. Kim, N. Kim, and S.G. Cho. The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. *Exp Mol Med* 2013;**45**:e2.

204 V. Mathews, A. Srivastava, and M. Chandy. Allogeneic stem cell transplantation for thalassemia major. *Hematol Oncol Clin North Am* 2014;**28**:1187–1200.

205 T. Zuckerman and J.M. Rowe. Transplantation in acute myeloid leukemia. *Hematol Oncol Clin North Am* 2014;**28**:983–994.

206 M.J. Ricci, J.A. Medin, and R.S. Foley. Advances in haplo-identical stem cell transplantation in adults with high-risk hematological malignancies. *World J Stem Cells* 2014;**6**:380–390.

207 E. Gluckman. Allogeneic transplantation strategies including haploidentical transplantation in sickle cell disease. *Hematol Am Soc Hematol Educ Program* 2013;**2013**:370–376.

208 T. Sugiyama, H. Kohara, M. Noda, and T. Nagasawa. Maintenance of the hematopoietic stem cell pool by CXCL12–CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006;**25**:977–988.

209 X. Cao, X. Wu, D. Frassica, *et al.* Irradiation induces bone injury by damaging bone marrow microenvironment for stem cells. *Proc Natl Acad Sci U S A* 2011;**108**:1609–1614.

210 K.H. Wu, H.P. Wu, C.K. Chan, *et al.* The role of mesenchymal stem cells in hematopoietic stem cell transplantation: from bench to bedsides. *Cell Transplant* 2013;**22**:723–729.

211 K. Murphy, P. Travers, and M. Walport. *Janeway's Immunology*, 7th edn. Garland Science, Taylor and Francis Group, 2008.

212 G.M. Spaggiari and L. Moretta. Cellular and molecular interactions of mesenchymal stem cells in innate immunity. *Immunol Cell Biol* 2013;**91**:27–31.

213 A.J. Nuata and W.E. Fibbe. Immunomodulatory properties of mesenchymal stem cells. *Blood* 2007;**110**:3499–3506.

214 K. English. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol* 2013;**91**:19–26.

215 M.M. Duffy, J. Pindjakova, S.A. Hanley, *et al.* Mesenchymal stem cell inhibition of T-helper 17 cell-differentiation is triggered by cell–cell contact and mediated by prostaglandin E2 via the EP4 receptor. *Eur J Immunol.* 2011;**41**:2840–2851.

216 M.M. Duffy, T. Ritter, R. Ceredig, and M.D. Griffin. Mesenchymal stem cell effects on T-cell effector pathways. *Stem Cell Res Ther* 2011;**2**:34.

217 P. Anklesaria, K. Kase, J. Glowacki, *et al.* Engraftment of a clonal bone marrow stromal cell line in vivo stimulates hematopoietic recovery from total body irradiation. *Proc Natl Acad Sci U S A* 1987;**84**:7681–7685.

218 O.N. Koç, S.L. Gerson, B.W. Cooper, *et al.* Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000;**18**:307–316.

219 Y. Mugurama, T. Yahata, H. Miyatake, *et al.* Reconstitution of the functional human hematopoietic microenvironment derived from human mesenchymal stem cells in the murine bone marrow compartment. *Blood* 2006;**107**:1878–1887.

220 M. L. Macmillan, B.R. Blazar, T.E. DeFor, and J.E. Wagner. Transplantation of *ex-vivo* culture-expanded parental haploidentical mesenchymal stem cells to promote engraftment in pediatric recipients of unrelated donor umbilical cord blood: results of a phase I-II clinical trial. *Bone Marrow Transplant* 2009;**43**:447–454.

221 S. Shim, S.B. Lee, G.-J. Lee, *et al.* Mitigating effects of hUCB-MSCs on the hematopoietic syndrome resulting from total body irradiation. *Exp Hematol* 2013;**41**:346–353.

222 S. François, M. Bensidhoum, M. Mouisedinne, *et al.* Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* 2006;**24**:1020–1029.

223 M. Mouiseddine, S. François, A. Semont, *et al.* Human mesenchymal stem cells home specifically to radiation-injured tissues in a non-obese diabetes/severe combined immunodeficiency mouse model. *Br J Radiol* 2007;**80**:S49–S55.

224 A. Chapel, J.M. Bertho, M. Bensidhoum, *et al.* Mesenchymal stem cells home to injured tissues when co-infused with hematopoietic cells to treat a radiation-induced multi-organ failure syndrome. *J Gene Med* 2003;**5**:1028–1038.

225 K. Le Blanc, I. Rasmusson, B. Sundberg, *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–1441.

226 K. Le Blanc, H. Samuelsson, B. Gustafsson, *et al.* Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* 2007;**21**:1733–1738.

227 P. Kebriaei, L. Isola, E. Bahceci, *et al.* Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. *Biol Blood Marrow Transplant* 2009;**15**: 804–811.

228 G. Lucchini, M. Introna, E. Dander, *et al.* Platelet-lysate-expanded mesenchymal stromal cells as a salvage therapy for severe resistant graft-versus-host disease in a pediatric population. *Biol Blood Marrow Transplant* 2010;**16**:1293–1301.

229 H.M. Lazarus, O.N. Koc, S.M. Devine, *et al.* Cotransplantation of HLA-identical sibling culture-expanded mesenchymal stem cells and hematopoietic stem cells in hematologic malignancy patients. *Biol Blood Marrow Transplant* 2005;**11**:389–398.

230 O. Ringden and K. LeBlanc. Mesenchymal stem cells for treatment of acute and chronic graft-versus-host disease, tissue toxicity and hemorrhages. *Best Pract Res Clin Haematol* 2011;**24**:65–72.

231 S.A. Bergfield and Y.A. DeClerck. Bone marrow-derived mesenchymal stem cells and the tumor microenvironment. *Cancer Metastasis Rev* 2010;**29**:249–261.

232 C. Schichor, T. Birnbaum, N. Etminan, *et al.* Vascular endothelial growth factor A contributes to glioma-induced migration of human marrow stromal cells (hMSC). *Exp Neurol* 2010;**199**:301–310.

233 I. Marigo and F. Dazzi. The immunomodulatory properties of mesenchymal stem cells. *Semin Immunopathol* 2011;**33**:593–602.

234 D. Hanahan and L.M. Coussens. Accessories to the crime: functions of cells recruited to the tumour microenvironment. *Cancer Cell* 2012;**21**:309–322.

235 S. Kidd, E. Spaeth, K. Watson, *et al.* Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma. *PLoS ONE* 2012;**7**:e30563.

236 M. Kraman, P.J. Brambrough, J.N. Arnold, *et al.* Suppression of anti-tumor immunity by stromal cells expressing fibroblast activation protein-α. *Science* 2010;**30**:827–830.

237 D. Hanahan and R.A. Weinberg. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–674.

238 B.G. Cuiffo and A.E. Karnoub. Mesenchymal stem cells in tumor development: emerging roles and concepts. *Cell Adh Migr* 2012;**6**:220–230.

239 P. Barcellos-de-Souza, V. Gori, F. Bambi, and P. Chiarugi. Tumor microenvironment: bone marrow-mesenchymal stem cells as key players. *Biochim Biophys Acta* 2013;**1836**:321–335.

240 M. Urashima, B.P. Chen, S. Chen, *et al.* The development of a model for the homing of multiple myeloma cells to human bone marrow. *Blood* 1997;**90**:754–765.

241 T. Michigami, N. Shimizu, P.J. Williams, *et al.* Cell–cell contact between marrow stromal cells and myeloma cells via VCAM-1 and $\alpha_4\beta_1$-integrin enhances production of osteoclast-stimulating activity. *Blood* 2000;**96**:1953–1960.

242 Y. Alsayed, H. Ngo, J. Runnels, *et al.* Mechanisms of regulation of CXCR4/SDF-1 (CXCL12)-dependent migration and homing in multiple myeloma. *Blood* 2007;**109**:2708–2717.

243 F. Ishikawa, S. Yoshida, Y. Saito, *et al.* Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol* 2007;**25**:1315–1321.

244 F. Ayala, R. Dewar, M. Kieran, and R. Kalluri. Contribution of bone microenvironment to leukemogenesis and leukemia progression. *Leukemia* 2009;**23**:2233–2241.

245 M. Ninomiya, A. Abe, A. Katsumi, *et al.* Homing, proliferation and survival sites of human leukemia cells *in vivo* in immunodeficient mice. *Leukemia* 2007;**21**:136–142.

246 A. Colmone, M. Amorim, A. L. Pontier, *et al.* Leukemic cells create bone marrow niches that disrupt the behavior of normal hematopoietic progenitor cells. *Science* 2008;**322**:1861–1865.

247 F. Nwajei and M. Konopleva. The bone marrow microenvironment as niche retreats for hematopoietic and leukemic stem cells. *Adv Hematol* 2013;**2013**:953–982.

248 K. Balakrishnan, J.A. Burger, M.P. Quiroga, *et al.* Influence of bone marrow stromal microenvironment on forodesine-induced responses in CLL primary cells. *Blood* 2010;**116**:1083–1091.

249 K. Balakrishnan, J.A. Burger, W.G. Wierda, and V. Gandhi. AT-101 induces apoptosis in CLL B cells and overcomes stromal cell-mediated Mcl-1 induction and drug resistance. *Blood* 2009;**113**: 149–153.

250 F. Vianello, F. Villanova, V. Tisato, *et al.* Bone marrow mesenchymal stromal cells non-selectively protect chronic myeloid leukemia cells from imatinib-induced apoptosis via the CXCR4/CXCL12 axis. *Haematologica* 2010;**95**:1081–1089.

251 J. Benito, Y. Shi, B. Szymanska, *et al.* Pronounced hypoxia in models of murine and human leukemia: high efficacy of hypoxia-activated prodrug PR-104. *PLoS ONE* 2011;**6**:e23108.

252 J.F. Fecteau, D. Messmer, S. Zhang, *et al.* Impact of oxygen concentration on growth of mesenchymal stromal cells from the marrow of patients with chronic lymphocytic leukemia. *Blood* 2013;**121**:971–974.

253 Y. Feng, M. Zhu, S. Dangelmajer, *et al.* Hypoxia-cultured human adipose-derived mesenchymal stem cells are non-oncogenic and have enhanced viability, motility, and tropism to brain cancer. *Cell Death Dis* 2014;**11**:e1567.

254 J. Tao, M.M. Jiang, L. Jiang, *et al.* Notch activation as an oncogenic driver of osteosarcoma. *Cancer Cell* 2014;**26**:390–401.

255 S.D. Berman, E. Calo, A.S. Landman, *et al.* Metastatic osteosarcoma induced by inactivation of Rb and p53 in the osteoblast lineage. *Proc Natl Acad Sci U S A* 2008;**105**:11851–11856.

256 J.R. Walkley, R. Qudsi, V.G. Sankaran, *et al.* Conditional mouse osteosarcoma, dependent on p53 loss and potentiated by loss of Rb, mimics the human disease. *Genes Dev* 2008;**22**:1662–1676.

257 D.D. Moore and R.C. Haydon. Ewing's sarcoma of bone. *Cancer Treat Res* 2014;**162**:93–115.

CHAPTER 28

# The implications of multipotent mesenchymal stromal cells in tumor biology and therapy

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## 28.1 Introduction

Multipotent mesenchymal stromal cells (MSCs) are able to differentiate *in vitro* into adipocytes, osteoblasts and chondrocytes [1] and into bone *in vivo*. MSCs have now been isolated from multiple organs and they form part of the endothelial wall of blood vessels [2,3]. In addition, they are a key component of tumor stroma and have been found to play important roles in various cancers [4]. Tumors, having many of the characteristics of injured tissue, may attract MSCs from their local tissue or from the circulation. The tumor tropism property of MSCs has suggested the possibility of their use as vehicles to deliver anticancer drugs or genes. However, extensive experimental research has demonstrated both anti- and procancer roles of MSCs in a context-dependent manner. It is now generally assumed that within this microenvironment reciprocal tumor–stroma crosstalk influences the phenotype of tumor cells, their progression, and their metastasis [5]. Various studies have reported the tumor-promoting effects of MSCs [6–8]. Others have provided evidence for an anti-oncogenic role of these cells [9–12]. MSCs are emerging as vehicles for anticancer drug/gene delivery [7]. This chapter aims to dissect this observed discrepancy in different experimental settings of human cancer so as to provide possible guidance to the appropriateness of clinical applications.

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## 28.2 Origin and identification of mesenchymal stromal cells in the tumor microenvironment

MSCs were initially identified by placing whole bone marrow cells in plastic culture dishes and observing the subsequent

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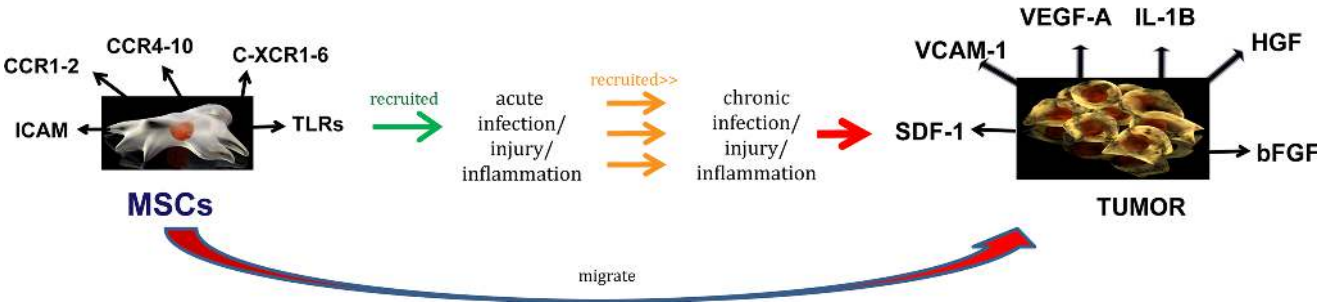



**Figure 28.1** Mechanisms for the recruitment of MSCs into tumors. MSCs express chemokine receptors, intercellular adhesion molecule (ICAM), C–C motif chemokine receptor (CCR)1–2, CCR4–10, C–X–C motif chemokine receptor (CXCR)1–6, and toll-like receptors (TLRs). Tumors release various cytokines, chemokines, and growth factors, including basic fibroblast growth factor (bFGF), stromal cell-derived factor 1 (SDF-1), hepatocyte growth factor (HGF), interleukin (IL)-1β, vascular cell adhesion molecule 1 (VCAM-1), and vascular endothelial growth factor (VEGF)-A, acting as chemoattractants for MSCs. MSCs in the local organ or tissue or circulating MSCs may be recruited into the tumor. MSCs may be constantly recruited in different stages from chronic infection, injury, and inflammation to cancer development.

expansion of a rare population of plastic-adherent cells [13,14]. The spatial distribution and properties of MSCs within their organ/tissue *in vivo* are relatively unclear [15]. MSCs have been isolated from most organs, including kidney, umbilical cord, brain, liver, lung, and bone marrow [16–18], and may have unique properties depending on their source. Recruited by the inflammatory milieu, MSCs migrate to the microenvironment of tumors and orchestrate the hallmarks of cancer cells [19]. The enrichment of MSCs in the tumor environment has been reported in both primary human cancers [20,21] and in metastases [22–24]. A resident population of MSCs has also been identified in the human adult liver and express a unique gene signature [25]. Additionally, MSCs share a molecular signature with mesenchymal tumor cells and favor early tumor growth in mice [26].

In response to injury or infection, MSCs can be released from the bone marrow and migrate toward sites of injury to promote tissue regeneration [27]. High frequencies of MSCs have been found in tumors with extensive inflammation, suggesting the recruitment of MSCs in response to inflammation [20]. Additionally, high circulating levels of endothelial progenitor cells have been observed in many cancer patients, which might also home to the site of tumors and promote tumor growth [28–30]. MSCs may be recruited locally and/or from the circulation to tumor sites (Figure 28.1). Future studies using somatic genomic signatures may provide a definite answer.

## 28.3 The migratory capacity of mesenchymal stromal cells

MSCs tend to be recruited by injured tissue, where they are thought to contribute to tissue repair and wound healing [31]. As tumors are often considered to have many characteristics of injured tissues, it is not surprising to find MSCs enriched within tumors. Various preclinical models have confirmed that MSCs can migrate to certain types of tumors, and this is one of the rationales of using MSCs as vehicles for anticancer drug/gene delivery [7,32]. MSCs are also relatively resistant to ischemia, because in the absence of oxygen they can survive by anaerobic adenosine triphosphate production [33], which may give them a competitive advantage in a tumor microenvironment. The tumor-tropic migratory property of MSCs is attributed to two main mechanisms [34,35].

### 28.3.1 Intrinsic migratory properties of mesenchymal stromal cells

Activated human MSCs express adhesion molecules such as integrins, ICAM-1, ICAM-2, and VCAM-1, which enable these cells to migrate [36]. MSCs also express chemokine receptors, including CCR1, CCR2, CCR4, CCR6, CCR7, CCR8, CCR9, CCR10, CXCR1, CXCR2, CXCR3, CXCR4, CXCR5, CXCR6, and CX3CR [31]. Production of their respective ligands is a characteristic of inflamed tissue and malignant tissue, and thus these receptors are likely involved in the specific accumulation of MSCs in both processes. Thus, the cognate ligands of these receptors are efficient chemotactic stimuli for MSCs. Additional receptors implicated in MSC migration are the TLRs. TLR1–6 have been identified in human MSCs and it has been reported that TLR stimulation enhanced the migratory function of MSCs [37].

### 28.3.2 Stimuli produced by the tumor

Malignant cells have been shown to produce relatively high amounts of MSC chemoattractants, including HGF, SDF-1 (also known as CXCL12), bFGF, VEGF-A, and VCAM-1 [38,39]. Tumor-derived IL-1β has been found to be a mediator of the proinflammatory response in MSCs exposed to tumor conditioned media, a mechanism that is regulated by focal adhesion kinase and mitogen-activated protein kinase signaling [40].

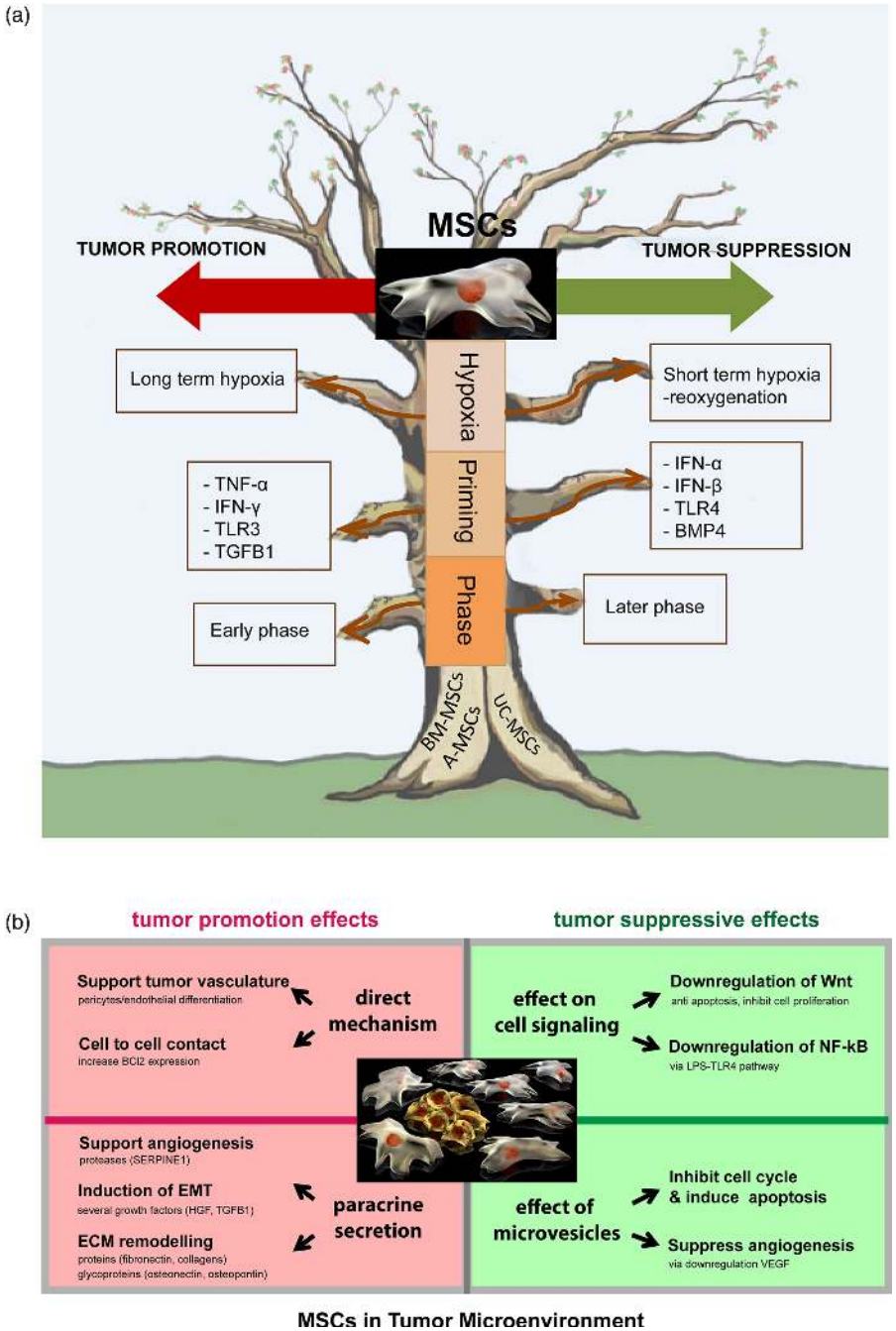



**Figure 28.2** Context-dependent role of MSCs in the tumor microenvironment. MSCs can exert tumor-promoting or tumor-limiting effects, which depend on the context in which the MSCs are present. (a) Different sources of MSCs, the presence of ligands, cytokine-primed MSCs, and the hypoxic microenvironment may lead to different effects of MSCs in the tumor microenvironment. The properties of the MSCs and the particular tumor microenvironment may result in dual roles for MSCs that can either suppress or promote tumor progression. (b) The tumor-suppressive mechanisms are mainly mediated by MSC microvesicles and downregulation of the Wnt and nuclear factor kappa light chain-enhancer of activated B cells (NF-κB) pathway. The tumor-promoting mechanisms are mainly attributed to factors secreted by MSCs and direct effects such as support of tumor vasculature, epithelial to mesenchymal transition (EMT) transition, and extracellular matrix (ECM) remodeling. BM-MSCs,: bone-marrow-derived MSCs; A-MSCs: adipose-derived MSCs; UC-MSCs: umbilical-cord-derived MSCs; BMP4: bone morphogenetic protein 4; IFN: interferon; LPS: lipopolysaccharide; TNF-α: tumor necrosis factor-α.

## 28.4 Context-dependent role of mesenchymal stromal cells in the tumor microenvironment

The tumor microenviroment is substantially different from that of normal organs and this is a mechanism in cancer development [41]. Depending on the experimental conditions, MSCs can exert tumor-promoting [20,24,42,43] or tumor-limiting effects [9,11,12,44,45]. Various hypotheses have been postulated to explain the context-dependent role of MSCs in cancer (Figure 28.2a).

### 28.4.1 Hypotheses on context-dependent roles of mesenchymal stromal cells in cancer

#### 28.4.1.1 Phase-dependent hypothesis

MSCs appear to promote tumor growth when co-injected with tumor cells, but inhibit tumor progression when administered into established tumors [4]. Thus, the presence of MSCs during

the early phase of tumorigenesis may contribute to angiogenesis that is required for tumor initiation. An increase in blood vessel density was observed when MSCs were co-injected with tumor cell lines [8,46].

#### 28.4.1.2 Priming-dependent hypothesis

MSCs express several TLRs, and their ability to migrate, invade, and secrete immune modulating factors is tightly regulated by specific TLR-agonist engagement [47]. TLR4-primed MSCs are polarized into a proinflammatory MSC type 1 (MSC1) phenotype, whereas TLR3-primed MSCs are polarized into the classical immunosuppressive MSC2 phenotype [47]. MSC1-based treatment of established tumors in an immune competent model attenuated tumor growth and metastasis, but MSC2-treated animals displayed increased tumor growth and metastasis [48]. The priming of all MSC types with inflammatory cytokines such as IFN-γ and TNF-α results in higher levels of VEGF [19] and induction of inhibition of runt-related transcription factor 2, one of the pivotal factors driving osteoblast differentiation [49]. These effects, in turn, can enhance tumor progression. However, stimulation of MSCs with IFN-α and IFN-β decreased tumor cell proliferation and induced tumor cell apoptosis in a mouse model of melanoma [50,51]. BMP4-differentiated bone marrow MSCs became less suppressive of T cell and natural killer (NK) cell proliferation and switched on their suppressive machinery by activating both indolamine 2,3-dioxygenase (IDO) and cyclooxygenase 2 (COX-2), promoting the differentiation of neighboring MSCs and triggering the anti-inflammatory effect [52]. In contrast, preconditioning of MSCs with TGF-β1 resulted in proinvasive MSCs in the progression of colon cancer [53].

#### 28.4.1.3 Hypoxic-dependent hypothesis

A long-term hypoxic microenvironment may lead to undifferentiated tumor cells and stromal cells, providing essential cellular interactions accompanied by the upregulation of the stemness genes [54,55]. Permanent hypoxia-stimulated MSCs proliferated and reduced their capacity to differentiate [56,57]. However, short-term oxygen limitation increased the number of apoptotic MSCs after 3–24 h of hypoxic treatment [58,59]. Moreover, oxygen limitation in hypoxia–reoxygenation-induced cell apoptosis was mediated in part by the reduction of phosphorylation of Akt and extracellular signal-regulated kinase 1/2 (ERK1/2) in MSCs [60]. ERK1/2 belongs to the class of protein kinase signal transduction pathways that are used to relay numerous extracellular signals within cells and have been reported to be involved in various cellular functions, including apoptosis and proliferation [61]. Hypoxia switches on various signaling pathways, and its context dependency determines the overall cell response and alterations in MSC functions.

Collectively, tumor cells and the tumor microenvironment will affect the ultimate function of recruited MSCs. However, there are some factors that drive MSCs to suppress or promote tumor growth (Figure 28.2b).

### 28.4.2 The tumor-suppressing roles of mesenchymal stromal cells

Extensive studies have reported tumor-suppressing effects of MSCs in various experimental cancer models. A variety of processes and mechanisms possibly implicated in MSC-dependent tumor suppression have been studied.

#### 28.4.2.1 Effect on cell signaling

Several signaling pathways have been reported to be associated with MSC suppression of tumor growth. Wnt signaling is aberrantly activated in many types of tumors. In chemically induced murine liver tumors the administration of MSCs has been demonstrated to have tumor suppressive effects associated with Wnt signaling. Its target genes were downregulated, especially those related to antiapoptosis, mitogenesis, cell proliferation, and cell cycle regulation [62]. MSCs can secrete Wnt inhibitors, such as Dickkopf-1 [52,63]. MSC-dependent inhibition of NF-κB signaling in cancer cells also occurs [64]. In addition, TLR signals can stimulate downstream effectors that may interfere with the LPS–TLR4 pathway and inhibit NF-κB activation during liver fibrosis [65].

#### 28.4.2.2 Effects of mesenchymal stromal cell microvesicles

Microvesicles are fragments of plasma membrane ranging from 100 to 1000 nm secreted by many cell types. They play an important role in intercellular communication and are capable of modifying the activity of target cells through surface receptor interactions and the transfer of proteins, mRNA, and microRNA (miRNA). Microvesicles have been implicated in tumor–stroma interactions [66]. Microvesicles released by MSCs have been associated with tumor inhibition in several preclinical studies [67] and have been shown to inhibit cell cycling and induce apoptosis or necrosis *in vitro* and to inhibit growth of established tumors *in vivo* [66,68,69], providing a further antioncogenic effect. Exosomes, a smaller type of intracellular vesicle derived from MSCs, suppress angiogenesis by downregulating VEGF expression in breast cancer cells [70], and MSCs pulsed with tumor-derived microvesicles exert an enhanced antitumor activity against hepatocellular cancer [71]. Thus, the secretome of MSCs appears to play an important role in their tumor suppressive function.

### 28.4.3 The tumor-promoting roles of mesenchymal stromal cells

The tumor-promoting role of MSCs has been attributed to direct mechanisms and paracrine secretion, including modulation of the immune response.

#### 28.4.3.1 Direct mechanisms

MSCs have been shown to directly differentiate into pericytes or possibly endothelial cells [72], thus supporting tumor angiogenesis, which in turn can promote tumor growth. MSC and

tumor cell contact is another important direct mechanism. In lymphoma models, direct cell–cell contact was the major mechanism of promoting tumor cell proliferation and survival rather than secretion of soluble factors by MSCs [73]. It is well recognized that adhesion to the bone marrow of the malignant cells of the B cell neoplasm, multiple myeloma, provides the myeloma cells with protection against chemotherapy. Bortezomib is a proteasome inhibitor used in the treatment of multiple myeloma and mantle cell lymphoma. MSCs can suppress bortezomib-induced multiple myeloma cell growth inhibition in a cell–cell contact-dependent manner by increasing Bcl2 expression in the myeloma cells [74]. Cell–cell contact with MSCs was reported to protect chronic lymphocytic leukemia cells, another B cell neoplasm, from spontaneous and drug-induced apoptosis [75]. Bone-marrow-derived MSCs have been reported to fuse with non-small cell lung cancer cells, resulting in highly malignant subpopulations with stem-cell-like properties [76].

#### 28.4.3.2 Paracrine mechanisms

MSC promotion of tumor growth via paracrine mechanisms is mainly attributed to supporting angiogenesis, promoting tumor growth, and metastasis. Secreted factors from patient tumor-derived MSCs have been shown to promote tumor growth in a xenograft mouse model associated with upregulation of cell growth and proliferation-related processes and downregulation of cell-death-related pathways in tumor cells [20]. Several growth factors secreted by MSCs, including HGF and TGF-β1, induce proinvasive signals in cancer [77,78]. A large number of proteases that have proangiogenic properties secreted by MSCs may inhibit apoptosis in vascular smooth muscle cells and endothelial cells [79]. The protease named serpin e1, a member of the serine proteinase inhibitor superfamily, is abundantly secreted by MSCs and has been shown to regulate proliferation, migration, and apoptosis of vascular smooth muscle cells and endothelial cells [80]. Transplantation of MSCs promoted microvascular growth in a mouse model [46,81], suggesting that angiogenesis plays an important role in support of tumor growth by MSCs.

A tumor-promoting effect attributed to remodeling of ECM via a paracrine secretion of MSCs also occurs. The ECM is a major component of the cellular microenvironment and is composed of diverse proteins such as collagens, elastins, fibronectin, proteoglycans, and glycoproteins [82]. Glycoproteins, such as osteonectin (also known as secreted protein acidic and rich in cysteine, SPARC), are highly expressed in stromal fibroblast cells that have been reported to promote tumor progression in several cancers [83–86]. Abundantly produced soluble fibronectin [83,87] by MSCs also plays an active role in the invasive process of human colon and liver cancer [88]. Matrix metalloproteinase proteases and hyaluronan, as well as various others factors secreted by MSCs, are capable of remodeling the ECM and facilitating tumor progression [20,89–91].

In contrast, human MSC-secreted microvesicles have been reported to have a striking antitumor effect in cancer [92] and tumor immune suppression [93], in which they transport mRNA, miRNA, and proteins between cells.

A functional role in neoplastic development and metastases has been attributed to the presence of miRNAs, small non-coding RNA molecules composed of approximately 22 nucleotides. They participate in RNA silencing and gene regulation [94]. In breast cancer, miR-21 and miR-205 were associated with tumor development, while miR-126 and miR-335 were related to metastases [37,95]. The promotion of progression of hepatocellular cancer by MSCs was attributed to miR-155 [89].

The effect of MSCs on tumor cells has also been reported to be associated with the induction of EMT [96], an effect that is further enhanced by the inflammatory milieu that characterizes many cancers. Initiation of metastasis requires invasion, and this is enabled by EMT: carcinoma cells in the primary tumor lose cell–cell adhesion mediated by repression of E-cadherin and break through the basement membrane with increased invasive properties and enter the bloodstream by intravasation. Later, when these circulating tumor cells exit the bloodstream to form micrometastases, they undergo the reverse process – mesothelial to epithelial transition. Evidence of a role for MSCs in EMT is the observation of increased expression of cancer-associated fibroblast and EMT markers in a co-culture model of hepatoma cells and MSCs [97]. There is also evidence that there are intricate links between EMT-type cells and drug resistance in tumors [98]. MSC-dependent EMT induction has been associated with shorter tumor-free survival and poorer overall survival, demonstrating the clinical relevance of this effect [96,99]. MSCs might also promote tumor progression or invasion via inducing regulation of secretion of IL-6 and secretion of SDF-1α in EMT [53,100].

## 28.5 The potential immunomodulation by mesenchymal stromal cells in the tumor microenvironment

MSCs can influence both the innate and adaptive immune systems, including the function of antigen-presenting cells [79,101], natural killer cells [102], B cells, and T cells [103,104] (and see Chapters 33 and 34). Immune suppressive cells accumulate in some tumors, which can impede immune surveillance and facilitate tumor growth [105]. The number and function of anti-tumor immune cells are decreased [54,106].

### 28.5.1 Mesenchymal stromal cells inhibit natural killer cells and macrophages

MSCs can modulate the function of NK cells [107] and macrophages [108]. NK cells are a type of lymphocyte that plays a role in the rejection of both tumors and virally infected cells. MSCs can inhibit the proliferation, cytotoxity, and cytokine production of NK cells through secretion of IDO and prostaglandin $E_2$ ($PGE_2$) [107,109]. In addition to IDO and $PGE_2$, cancer-

associated stromal cells produce other soluble factors, such as VEGF and platelet-derived growth factor, which enhance tumor progression by promoting or attracting M2 macrophages, which are characterized by their production of high levels of the suppressive cytokine IL-10 and low levels of the proinflammatory cytokines IFN-γ and TNF-α [110]. M2 macrophages activate T helper 2 cell activity and also promote angiogenesis, tissue remodeling, and repair. Proinflammatory stimulation by IFN-γ, TNF-α, or LPS increases the expression of COX-2 and IDO in MSCs and these enzymes promote further M2 macrophage polarization [108]. Macrophages also play an important role in the innate immune response to virus infections. Innate immune responses, including TLRs, are important for viral clearance [111].

### 28.5.2 Mesenchymal stromal cells inhibit T cell proliferation

T cells are the major player of the adaptive immune response and are important in controlling malignant disease, mediating both cytotoxicity of cancer cells and releasing anti-oncogenic cytokines [112,113]. MSCs can inhibit T cell function through multiple pathways [114,115]. MSC suppression of T cell responses can be mediated by cell contact and soluble factors, including TGF-β, HGF, $PGE_2$, soluble human leukocyte antigen (HLA)-G5, IDO, and inducible nitric oxide synthetase [112,113,116–118]. Inhibition of T cell function by MSCs affects T cell proliferation and IFN-γ production and induces the production of IL-4, resulting in a shift from a proinflammatory T cells to anti-inflammatory T cells [114,115]. Cell–cell contact T cell inhibition by MSCs can be mediated by surface expression of HLA-G [119], a nonclassical major histocompatibility complex class I molecule with tolerogenic functions that contribute to fetal graft tolerance and human allograft acceptance [120]. Fas ligand and programmed death-ligand 1 also play significant roles in immunomodulation mediated by MSCs [121].

### 28.5.3 Mesenchymal stromal cells promote the expansion and function of regulatory T cells

Regulatory T cells (Tregs) are a subset of T cells that suppress activation of the immune system to maintain homeostasis and tolerance to self-antigens. An increased number of highly activated Tregs were found to infiltrate the tumor milieu of liver tumors in which they were mainly localized in the stromal compartment of the tumors [106]. The frequency of Tregs has been associated with poor prognosis [122–124]. MSCs can induce the generation and expansion of Tregs by the secretion of TGF-β [125], IDO [126] and the release of soluble HLA-G5 [113]. In contrast to their suppression of cytotoxic T cells, MSCs can induce the generation and expansion of Tregs [127]. Additionally, MSCs have been reported to induce the production of IL-10 by plasmacytoid dendritic cells (DCs), which in turn triggered the generation of Tregs [114].

### 28.5.4 Mesenchymal stromal cells inhibit the function of dendritic cells

It has been found that MSCs also display immunosuppressive potential through inhibiting the differentiation of DCs. DCs are the most efficient cells in presenting antigen to T cells. They play a key role in the initiation of the primary immune responses and in tolerance, depending on their activation and maturation status [128]. MSCs are capable of modulating the differentiation, activation, and function of DCs [129]. MSCs reduce the production of several cytokines by DCs, including IL-12 and TNF-α [130]. MSCs isolated from different tissue sources present distinct immunomodulatory profiles [131], so it will be important to more closely study MSCs present in tumors.

## 28.6 Therapeutic application of mesenchymal stromal cells in cancer

### 28.6.1 Potential therapeutic application

Several studies have demonstrated that MSCs have the capacity to reverse acute and chronic injury in different experimental settings [132–135]. MSCs have been reported to attenuate inflammation [136–138] and ameliorate autoimmune diseases [10,139–141]. The fact that MSCs can migrate into certain types of tumors has led to their use as vehicles for tumor-specific delivery of anticancer drugs or genes.

Genetically modified MSCs have been used to deliver anticancer genes and inhibit cancer cell proliferation *in vitro* and *in vivo* [95,142–144]; and see Chapter 62. Several studies have demonstrated that MSCs have anticancer effects in different experimental settings [62,96,145–147]. MSCs have been extensively investigated in clinical trials as potential therapy in a number of different diseases [148,149]. Approximately 18 trials have been registered at ClinicalTrials.gov involving the use of MSCs in various cancers. These include ovarian cancer (NCT02068794), prostate cancer (NCT01983709), and hematologic malignancy (NCT01854567).

### 28.6.2 Reasons for caution

Given the context-dependent role of MSCs, it appears possible that MSCs in the tumor microenvironment could promote tumor growth [150]. MSCs may facilitate malignant development in patients at high risk of developing cancer, such as those with chronic hepatitis B or C patients or recipients of organ transplants [19,151,152]. In addition, MSCs have the potential for malignant transformation during *ex vivo* expansion [153].

Furthermore, although MSCs can be detected by magnetic resonance imaging or radioactive labeling [154] for up to 25 days [25], the cellular fate and distribution *in vivo* of transplanted MSCs remain unclear because techniques for tracking infused MSCs have low sensitivity [155]. Because of unclear clinical benefits of MSCs in patients with cancer [156–158] and because of the role of MSCs in the tumor microenvironment, it would be

wise to be cautious in the use of MSCs in patients with malignant diseases.

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## References

1 A. J. Friedenstein, S. Piatetzky, II, K. V. Petrakova. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* 1966;**16**:381–390.
2 C. M. Kolf, E. Cho, R. S. Tuan. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* 2007;**9**:204.
3 D. G. Phinney, D. J. Prockop. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* 2007;**25**:2896–2902.
4 A. H. Klopp, A. Gupta, E. Spaeth, *et al*. Concise review: dissecting a discrepancy in the literature: do mesenchymal stem cells support or suppress tumor growth? *Stem Cells* 2011;**29**:11–19.
5 D. Hanahan, R. A. Weinberg. Hallmarks of cancer: the next generation. *Cell* 2011;**144**:646–674.
6 A. E. Karnoub, A. B. Dash, A. P. Vo, *et al*. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**:557–563.
7 Z. Gao, L. Zhang, J. Hu, Y. Sun. Mesenchymal stem cells: a potential targeted-delivery vehicle for anti-cancer drug, loaded nanoparticles. *Nanomedicine* 2013;**9**:174–184.
8 B. M. Beckermann, G. Kallifatidis, A. Groth, *et al*. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer* 2008;**99**:622–631.
9 G. C. Li, Q. H. Ye, Y. H. Xue, *et al*. Human mesenchymal stem cells inhibit metastasis of a hepatocellular carcinoma model using the MHCC97-H cell line. *Cancer Sci* 2010;**101**:2546–2553.
10 J. Y. Oh, T. W. Kim, H. J. Jeong, *et al*. Intraperitoneal infusion of mesenchymal stem/stromal cells prevents experimental autoimmune uveitis in mice. *Mediators Inflamm* 2014;**2014**:624640.
11 W. Zhao, G. Ren, L. Zhang, *et al*. Efficacy of mesenchymal stem cells derived from human adipose tissue in inhibition of hepatocellular carcinoma cells *in vitro*. *Cancer Biother Radiopharm* 2012;**27**:606–613.
12 K. Takahara, M. Ii, T. Inamoto, *et al*. Adipose-derived stromal cells inhibit prostate cancer cell proliferation inducing apoptosis. *Biochem Biophys Res Commun* 2014;**446**:1102–1107.
13 A. J. Friedenstein, R. K. Chailakhjan, K. S. Lalykina. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**:393–403.
14 E. M. Horwitz, K. Le Blanc, M. Dominici, *et al*. Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 2005;**7**:393–395.
15 P. Bianco, P. G. Robey, P. J. Simmons. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008;**2**:313–319.
16 L. da Silva Meirelles, P. C. Chagastelles, N. B. Nardi. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**:2204–2213.
17 P. A. Zuk, M. Zhu, H. Mizuno, *et al*. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**:211–228.
18 H. Y. Choi, S. J. Moon, B. B. Ratliff, *et al*. Microparticles from kidney-derived mesenchymal stem cells act as carriers of proangiogenic signals and contribute to recovery from acute kidney injury. *PLoS ONE* 2014;**9**:e87853.
19 Y. Liu, Z. P. Han, S. S. Zhang, *et al*. Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. *J Biol Chem* 2011;**286**:25007–25015.
20 P. Y. Hernanda, A. Pedroza-Gonzalez, L. J. van der Laan, *et al*. Tumor promotion through the mesenchymal stem cell compartment in human hepatocellular carcinoma. *Carcinogenesis* 2013;**34**:2330–2340.
21 F. Huang, M. Wang, T. Yang, *et al*. Gastric cancer-derived MSC-secreted PDGF-DD promotes gastric cancer progression. *J Cancer Res Clin Oncol* 2014;**140**:1835–1848.
22 P. Hernanda, A. Pedroza-Gonzales, L. J. W. van der Laan, *et al*. Human liver carcinomas recruit mesenchymal stem/stromal cells that can promote tumor growth via paracrine signaling. *J Hepatol* 2013;**58**:S431.
23 H. Li, Z. Feng, T. C. Tsang, *et al*. Fusion of HepG2 cells with mesenchymal stem cells increases cancer-associated and malignant properties: an *in vivo* metastasis model. *Oncol Rep* 2014;**32**:539–547.
24 B. Song, B. Kim, S. H. Choi, *et al*. Mesenchymal stromal cells promote tumor progression in fibrosarcoma and gastric cancer cells. *Korean J Pathol* 2014;**48**:217–224.
25 Q. Pan, S. M. Fouraschen, F. S. Kaya, *et al*. Mobilization of hepatic mesenchymal stem cells from human liver grafts. *Liver Transpl* 2011;**17**:596–609.
26 M. Galie, G. Konstantinidou, D. Peroni, *et al*. Mesenchymal stem cells share molecular signature with mesenchymal tumor cells and favor early tumor growth in syngeneic mice. *Oncogene* 2008;**27**:2542–2551.
27 E. Mansilla, G. H. Marin, H. Drago, *et al*. Bloodstream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. *Transplant Proc* 2006;**38**:967–969.
28 C. C. Ling, K. T. Ng, Y. Shao, *et al*. Post-transplant endothelial progenitor cell mobilization via CXCL10/CXCR3 signaling promotes liver tumor growth. *J Hepatol* 2014;**60**:103–109.
29 A. Taddeo, P. Presicce, L. Brambilla, *et al*. Circulating endothelial progenitor cells are increased in patients with classic Kaposi's sarcoma. *J Invest Dermatol* 2008;**128**:2125–2128.
30 C. F. Bellows, Y. Zhang, J. Chen, *et al*. Circulation of progenitor cells in obese and lean colorectal cancer patients. *Cancer Epidemiol Biomarkers Prev* 2011;**20**:2461–2468.
31 J. Ringe, S. Strassburg, K. Neumann, *et al*. Towards *in situ* tissue repair: human mesenchymal stem cells express chemokine

receptors CXCR1, CXCR2 and CCR2, and migrate upon stimulation with CXCL8 but not CCL2. *J Cell Biochem* 2007;**101**:135–146.
32 L. J. Dai, M. R. Moniri, Z. R. Zeng, *et al.* Potential implications of mesenchymal stem cells in cancer therapy. *Cancer Lett* 2011;**305**:8–20.
33 L. A. Mylotte, A. M. Duffy, M. Murphy, *et al.* Metabolic flexibility permits mesenchymal stem cell survival in an ischemic environment. *Stem Cells* 2008;**26**:1325–1336.
34 R. N. Kaplan, B. Psaila, D. Lyden. Niche-to-niche migration of bone-marrow-derived cells. *Trends Mol Med* 2007;**13**:72–81.
35 P. Y. Hernanda, A. Pedroza-Gonzalez, D. Sprengers, *et al.* Multipotent mesenchymal stromal cells in liver cancer: implications for tumor biology and therapy. *Biochim Biophys Acta* 2014;**1846**: 439–445.
36 G. Xu, L. Zhang, G. Ren, *et al.* Immunosuppressive properties of cloned bone marrow mesenchymal stem cells. *Cell Res* 2007;**17**: 240–248.
37 M. Tome, P. López-Romero, C. Albo, *et al.* miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. *Cell Death Differ* 2011;**18**:985–995.
38 A. Schmidt, D. Ladage, T. Schinkothe, *et al.* Basic fibroblast growth factor controls migration in human mesenchymal stem cells. *Stem Cells* 2006;**24**:1750–1758.
39 M. G. Garcia, J. Bayo, M. F. Bolontrade, *et al.* Hepatocellular carcinoma cells and their fibrotic microenvironment modulate bone marrow-derived mesenchymal stromal cell migration *in vitro* and *in vivo*. *Mol Pharm* 2011;**8**:1538–1548.
40 M. Al-toub, A. Almusa, M. Almajed, *et al.* Pleiotropic effects of cancer cells' secreted factors on human stromal (mesenchymal) stem cells. *Stem Cell Res Ther* 2013;**4**:114.
41 L. E. Holz, G. W. McCaughan, V. Benseler, *et al.* Liver tolerance and the manipulation of immune outcomes. *Inflamm Allergy Drug Targets* 2008;**7**:6–18.
42 N. Bezdenezhnykh, N. Semesiuk, O. Lykhova, *et al.* Impact of stromal cell components of tumor microenvironment on epithelial–mesenchymal transition in breast cancer cells. *Exp Oncol* 2014;**36**:72–78.
43 T. Li, B. Song, X. Du, *et al.* Effect of bone-marrow-derived mesenchymal stem cells on high-potential hepatocellular carcinoma in mouse models: an intervention study. *Eur J Med Res* 2013;**18**:34.
44 H. Ryu, J. E. Oh, K. J. Rhee, *et al.* Adipose tissue-derived mesenchymal stem cells cultured at high density express IFN-β and suppress the growth of MCF-7 human breast cancer cells. *Cancer Lett* 2014;**352**:220–227.
45 S. H. Abd-Allah, S. M. Shalaby, A. S. El-Shal, *et al.* Effect of bone marrow-derived mesenchymal stromal cells on hepatoma. *Cytotherapy* 2014;**16**:1197–1206.
46 P. Gong, Y. Wang, Y. Wang, *et al.* Effect of bone marrow mesenchymal stem cells on hepatocellular carcinoma in microcirculation. *Tumour Biol* 2013;**34**:2161–2168.
47 R. S. Waterman, S. L. Tomchuck, S. L. Henkle, A. M. Betancourt. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* 2010;**5**:e10088.
48 R. S. Waterman, S. L. Henkle, A. M. Betancourt. Mesenchymal stem cell 1 (MSC1)-based therapy attenuates tumor growth whereas MSC2-treatment promotes tumor growth and metastasis. *PLoS ONE* 2012;**7**:e45590.
49 J. S. Lee, J. M. Lee, G. I. Im. Electroporation-mediated transfer of Runx2 and Osterix genes to enhance osteogenesis of adipose stem cells. *Biomaterials* 2011;**32**:760–768.
50 J. Ahn, H. Lee, K. Seo, *et al.* Anti-tumor effect of adipose tissue derived-mesenchymal stem cells expressing interferon-β and treatment with cisplatin in a xenograft mouse model for canine melanoma. *PLoS ONE* 2013;**8**:e74897.
51 C. Xu, L. Lin, G. Cao, *et al.* Interferon-α-secreting mesenchymal stem cells exert potent antitumor effect *in vivo*. *Oncogene* 2014;**33**:5047–5052.
52 G. Bassi, F. Guilloton, C. Menard, *et al.* Effects of a ceramic biomaterial on immune modulatory properties and differentiation potential of human mesenchymal stromal cells of different origin. *Tissue Eng Part A* 2015;**21**(3–4):767–781.
53 A. De Boeck, A. Hendrix, D. Maynard, *et al.* Differential secretome analysis of cancer-associated fibroblasts and bone marrow-derived precursors to identify microenvironmental regulators of colon cancer progression. *Proteomics* 2013;**13**:379–388.
54 Q. Lin, Z. Yun. Impact of the hypoxic tumor microenvironment on the regulation of cancer stem cell characteristics. *Cancer Biol Ther* 2010;**9**:949–956.
55 A. Lavrentieva, I. Majore, C. Kasper, R. Hass. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. *Cell Commun Signal* 2010;**8**:18.
56 L. B. Buravkova, Y. V. Rylova, E. R. Andreeva, *et al.* Low ATP level is sufficient to maintain the uncommitted state of multipotent mesenchymal stem cells. *Biochim Biophys Acta* 2013;**1830**:4418–4425.
57 A. Salim, R. P. Nacamuli, E. F. Morgan, *et al.* Transient changes in oxygen tension inhibit osteogenic differentiation and Runx2 expression in osteoblasts. *J Biol Chem* 2004;**279**:40007–40016.
58 W. Zhu, J. Chen, X. Cong, *et al.* Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Stem Cells* 2006;**24**:416–425.
59 Y. Nie, B. M. Han, X. B. Liu, *et al.* Identification of microRNAs involved in hypoxia- and serum deprivation-induced apoptosis in mesenchymal stem cells. *Int J Biol Sci* 2011;**7**:762–768.
60 J. A. Wang, T. L. Chen, J. Jiang, *et al.* Hypoxic preconditioning attenuates hypoxia/reoxygenation-induced apoptosis in mesenchymal stem cells. *Acta Pharmacol Sin* 2008;**29**:74–82.
61 G. D. Dispersyn, M. Borgers. Apoptosis in the heart: about programmed cell death and survival. *News Physiol Sci* 2001;**16**:41–47.
62 M. T. Abdel aziz, M. F. El Asmar, H. M. Atta, *et al.* Efficacy of mesenchymal stem cells in suppression of hepatocarcinorigenesis in rats: possible role of Wnt signaling. *J Exp Clin Cancer Res* 2011;**30**:49.
63 L. Qiao, Z. Xu, T. Zhao, *et al.* Suppression of tumorigenesis by human mesenchymal stem cells in a hepatoma model. *Cell Res* 2008;**18**:500–507.
64 L. Qiao, T. J. Zhao, F. Z. Wang, *et al.* NF-κB downregulation may be involved the depression of tumor cell proliferation mediated by human mesenchymal stem cells. *Acta Pharmacol Sin* 2008;**29**: 333–340.
65 P. P. Wang, D. Y. Xie, X. J. Liang, *et al.* HGF and direct mesenchymal stem cells contact synergize to inhibit hepatic stellate cells activation through TLR4/NF-kB pathway. *PLoS ONE* 2012;**7**: e43408.
66 S. Bruno, F. Collino, M. C. Deregibus, *et al.* Microvesicles derived from human bone marrow mesenchymal stem cells inhibit tumor growth. *Stem Cells Dev* 2013;**22**:758–771.

67 C. Akyurekli, Y. Le, R. B. Richardson, *et al.* A systematic review of preclinical studies on the therapeutic potential of mesenchymal stromal cell-derived microvesicles. *Stem Cell Rev* 2015;**11**(1): 150–160.

68 R. D. Balsara, V. A. Ploplis. Plasminogen activator inhibitor-1: the double-edged sword in apoptosis. *Thromb Haemost* 2008;**100**: 1029–1036.

69 Y. Zhang, L. Li, J. Yu, *et al.* Microvesicle-mediated delivery of transforming growth factor beta1 siRNA for the suppression of tumor growth in mice. *Biomaterials* 2014;**35**:4390–4400.

70 J. K. Lee, S. R. Park, B. K. Jung, *et al.* Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* 2013;**8**:e84256.

71 B. Ma, H. Jiang, J. Jia, *et al.* Murine bone marrow stromal cells pulsed with homologous tumor-derived exosomes inhibit proliferation of liver cancer cells. *Clin Transl Oncol* 2012;**14**:764–773.

72 A. Al-Khaldi, N. Eliopoulos, D. Martineau, *et al.* Postnatal bone marrow stromal cells elicit a potent VEGF-dependent neoangiogenic response *in vivo*. *Gene Ther* 2003;**10**:621–629.

73 B. D. Roorda, A. Elst, T. G. Boer, *et al.* Mesenchymal stem cells contribute to tumor cell proliferation by direct cell–cell contact interactions. *Cancer Invest* 2010;**28**:526–534.

74 M. Hao, L. Zhang, G. An, *et al.* Bone marrow stromal cells protect myeloma cells from bortezomib induced apoptosis by suppressing microRNA-15a expression. *Leuk Lymphoma* 2011;**52**:1787–1794.

75 A. V. Kurtova, K. Balakrishnan, R. Chen, *et al.* Diverse marrow stromal cells protect CLL cells from spontaneous and drug-induced apoptosis: development of a reliable and reproducible system to assess stromal cell adhesion-mediated drug resistance. *Blood* 2009;**114**:4441–4450.

76 M. H. Xu, X. Gao, D. Luo, *et al.* EMT and acquisition of stem cell-like properties are involved in spontaneous formation of tumorigenic hybrids between lung cancer and bone marrow-derived mesenchymal stem cells. *PLoS ONE* 2014;**9**:e87893.

77 O. De Wever, Q. D. Nguyen, L. Van Hoorde, *et al.* Tenascin-C and SF/HGF produced by myofibroblasts in vitro provide convergent pro-invasive signals to human colon cancer cells through RhoA and Rac. *FASEB J* 2004;**18**:1016–1018.

78 M. J. Lee, S. C. Heo, S. H. Shin, *et al.* Oncostatin M promotes mesenchymal stem cell-stimulated tumor growth through a paracrine mechanism involving periostin and TGFBI. *Int J Biochem Cell Biol* 2013;**45**:1869–1877.

79 A. Uccelli, L. Moretta, V. Pistoia. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;**8**:726–736.

80 N. M. Hogan, M. R. Joyce, J. M. Murphy, *et al.* Impact of mesenchymal stem cell secreted PAI-1 on colon cancer cell migration and proliferation. *Biochem Biophys Res Commun* 2013;**435**:574–579.

81 M. S. Ko, J. Y. Jung, I. S. Shin, *et al.* Effects of expanded human adipose tissue-derived mesenchymal stem cells on the viability of cryopreserved fat grafts in the nude mouse. *Int J Med Sci* 2011;**8**:231–238.

82 W. P. Daley, S. B. Peters, M. Larsen. Extracellular matrix dynamics in development and regenerative medicine. *J Cell Sci* 2008;**121**: 255–264.

83 B. Le Bail, S. Faouzi, L. Boussarie, *et al.* Osteonectin/SPARC is overexpressed in human hepatocellular carcinoma. *J Pathol* 1999; **189**:46–52.

84 N. Yusuf, T. Inagaki, S. Kusunoki, *et al.* SPARC was overexpressed in human endometrial cancer stem-like cells and promoted migration activity. *Gynecol Oncol* 2014;**134**:356–363.

85 C. Neuzillet, A. Tijeras-Raballand, J. Cros, *et al.* Stromal expression of SPARC in pancreatic adenocarcinoma. *Cancer Metastasis Rev* 2013;**32**:585–602.

86 S. Sangaletti, C. Tripodo, P. Portararo, *et al.* Stromal niche communalities underscore the contribution of the matricellular protein SPARC to B-cell development and lymphoid malignancies. *Oncoimmunology* 2014;**3**:e28989.

87 J. Ou, Y. Peng, J. Deng, *et al.* Endothelial cell-derived fibronectin extra domain A promotes colorectal cancer metastasis via inducing epithelial–mesenchymal transition. *Carcinogenesis* 2014;**35**: 1661–1670.

88 B. Tian, Y. Li, X. N. Ji, *et al.* Basement membrane proteins play an active role in the invasive process of human hepatocellular carcinoma cells with high metastasis potential. *J Cancer Res Clin Oncol* 2005;**131**:80–86.

89 X. L. Yan, Y. L. Jia, L. Chen, *et al.* Hepatocellular carcinoma-associated mesenchymal stem cells promote hepatocarcinoma progression: role of the S100A4–miR155–SOCS1–MMP9 axis. *Hepatology* 2013;**57**:2274–2286.

90 A. Calabro, M. M. Oken, V. C. Hascall, A. M. Masellis. Characterization of hyaluronan synthase expression and hyaluronan synthesis in bone marrow mesenchymal progenitor cells: predominant expression of HAS1 mRNA and up-regulated hyaluronan synthesis in bone marrow cells derived from multiple myeloma patients. *Blood* 2002;**100**:2578–2585.

91 N. Tobar, M. C. Avalos, N. Mendez, *et al.* Soluble MMP-14 produced by bone marrow-derived stromal cells sheds epithelial endoglin modulating the migratory properties of human breast cancer cells. *Carcinogenesis* 2014;**35**:1770–1779.

92 D. Castellana, F. Zobairi, M. C. Martinez, *et al.* Membrane microvesicles as actors in the establishment of a favorable prostatic tumoral niche: a role for activated fibroblasts and CX3CL1–CX3CR1 axis. *Cancer Res* 2009;**69**:785–793.

93 P. D. Robbins, A. E. Morelli. Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* 2014;**14**:195–208.

94 D. P. Bartel. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004;**116**:281–297.

95 S. F. Tavazoie, C. Alarcón, T. Oskarsson, *et al.* Endogenous human microRNAs that suppress breast cancer metastasis. *Nature* 2008;**451**:147–152.

96 Y. Jing, Z. Han, Y. Liu, *et al.* Mesenchymal stem cells in inflammation microenvironment accelerates hepatocellular carcinoma metastasis by inducing epithelial–mesenchymal transition. *PLoS ONE* 2012;**7**:e43272.

97 S. D. Bhattacharya, Z. Mi, L. J. Talbot, *et al.* Human mesenchymal stem cell and epithelial hepatic carcinoma cell lines in admixture: concurrent stimulation of cancer-associated fibroblasts and epithelial-to-mesenchymal transition markers. *Surgery* 2012;**152**: 449–454.

98 H. Sui, L. Zhu, W. Deng, Q. Li. Epithelial–mesenchymal transition and drug resistance: role, molecular mechanisms, and therapeutic strategies. *Oncol Res Treat* 2014;**37**:584–589.

99 S. Saito, H. Okabe, M. Watanabe, *et al.* CD44v6 expression is related to mesenchymal phenotype and poor prognosis in patients with colorectal cancer. *Oncol Rep* 2013;**29**:1570–1578.

100 E. L. Spaeth, J. L. Dembinski, A. K. Sasser, *et al.* Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS ONE* 2009;**4**:e4992.

101 X. X. Jiang, Y. Zhang, B. Liu, *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**:4120–4126.

102 P. A. Sotiropoulou, S. A. Perez, A. D. Gritzapis, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**:74–85.

103 A. Corcione, F. Benvenuto, E. Ferretti, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**:367–372.

104 F. Benvenuto, S. Ferrari, E. Gerdoni, *et al.* Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* 2007;**25**:1753–1760.

105 R. Hass, A. Otte. Mesenchymal stem cells as all-round supporters in a normal and neoplastic microenvironment. *Cell Commun Signal* 2012;**10**:26.

106 A. Pedroza-Gonzalez, C. Verhoef, J. N. Ijzermans, *et al.* Activated tumor-infiltrating $CD4^+$ regulatory T cells restrain antitumor immunity in patients with primary or metastatic liver cancer. *Hepatology* 2013;**57**:183–194.

107 C. Noone, A. Kihm, K. English, *et al.* IFN-γ stimulated human umbilical-tissue-derived cells potently suppress NK activation and resist NK-mediated cytotoxicity *in vitro*. *Stem Cells Dev* 2013;**22**:3003–3014.

108 M. Francois, R. Romieu-Mourez, M. Li, L. Galipeau. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther* 2012;**20**:187–195.

109 G. M. Spaggiari, A. Capobianco, H. Abdelrazik, *et al.* Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008;**111**:1327–1333.

110 J. Maggini, G. Mirkin, I. Bognanni, *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE* 2010;**5**:e9252.

111 N. Arpaia, G. M. Barton. Toll-like receptors: key players in antiviral immunity. *Curr Opin Virol* 2011;**1**:447–454.

112 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.

113 Z. Selmani, A. Naji, I. Zidi, *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**:212–222.

114 S. Aggarwal, M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.

115 E. Zappia, S. Casazza, E. Pedemonte, *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 2005;**106**:1755–1761.

116 K. Sato, K. Ozaki, I. Oh, *et al.* Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* 2007;**109**:228–234.

117 R. Meisel, A. Zibert, M. Laryea, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**:4619–4621.

118 A. Nasef, N. Mathieu, A. Chapel, *et al.* Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation* 2007;**84**:231–237.

119 M. Giuliani, M. Fleury, A. Vernochet, *et al.* Long-lasting inhibitory effects of fetal liver mesenchymal stem cells on T-lymphocyte proliferation. *PLoS ONE* 2011;**6**:e19988.

120 E. D. Carosella, P. Moreau, J. Le Maoult, *et al.* HLA-G molecules: from maternal–fetal tolerance to tissue acceptance. *Adv Immunol* 2003;**81**:199–252.

121 Y. Z. Gu, Q. Xue, Y. J. Chen, *et al.* Different roles of PD-L1 and FasL in immunomodulation mediated by human placenta-derived mesenchymal stem cells. *Hum Immunol* 2013;**74**:267–276.

122 X. Shen, N. Li, H. Li, *et al.* Increased prevalence of regulatory T cells in the tumor microenvironment and its correlation with TNM stage of hepatocellular carcinoma. *J Cancer Res Clin Oncol* 2010;**136**:1745–1754.

123 K. J. Chen, S. Z. Lin, L. Zhou, *et al.* Selective recruitment of regulatory T cell through CCR6–CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. *PLoS ONE* 2011;**6**:e24671.

124 S. Z. Lin, K. J. Chen, Z. Y. Xu, *et al.* Prediction of recurrence and survival in hepatocellular carcinoma based on two Cox models mainly determined by $FoxP3^+$ regulatory T cells. *Cancer Prev Res (Phila)* 2013;**6**:594–602.

125 S. A. Patel, J. R. Meyer, S. J. Greco, *et al.* Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-β. *J Immunol* 2010;**184**:5885–5894.

126 W. Ge, J. Jiang, J. Arp, *et al.* Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* 2010;**90**:1312–1320.

127 X. Xia, W. Chen, T. Ma, *et al.* Mesenchymal stem cells administered after liver transplantation prevent acute graft-versus-host disease in rats. *Liver Transpl* 2012;**18**:696–706.

128 R. M. Steinman, J. Banchereau. Taking dendritic cells into medicine. *Nature* 2007;**449**:419–426.

129 Z. Han, Y. Jing, S. Zhang, *et al.* The role of immunosuppression of mesenchymal stem cells in tissue repair and tumor growth. *Cell Biosci* 2012;**2**:8.

130 W. Zhang, W. Ge, C. Li, *et al.* Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. *Stem Cells Dev* 2004;**13**:263–271.

131 M. Najar, G. Raicevic, H. Fayyad-Kazan, *et al.* Impact of different mesenchymal stromal cell types on T-cell activation, proliferation and migration. *Int Immunopharmacol* 2013;**15**:693–702.

132 A. Monsel, Y. G. Zhu, S. Gennai, *et al.* Cell-based therapy for acute organ injury: preclinical evidence and ongoing clinical trials using mesenchymal stem cells. *Anesthesiology* 2014;**121**:1099–1121.

133 V. Donega, C. H. Nijboer, L. Braccioli, *et al.* Intranasal administration of human MSC for ischemic brain injury in the mouse: *in vitro* and *in vivo* neuroregenerative functions. *PLoS ONE* 2014;**9**:e112339.

134 Y. Zhang, W. Cai, Q. Huang, *et al.* Mesenchymal stem cells alleviate bacteria-induced liver injury in mice by inducing regulatory dendritic cells. *Hepatology* 2014;**59**:671–682.

135 K. H. Jung, T. Yi, M. K. Son, *et al.* Therapeutic effect of human clonal bone marrow-derived mesenchymal stem cells in severe acute pancreatitis. *Arch Pharm Res* 2015;**38**(5):742–751.

136 K. Raza, T. Larsen, N. Samaratunga, *et al.* MSC therapy attenuates obliterative bronchiolitis after murine bone marrow transplant. *PLoS ONE* 2014;**9**:e109034.

137 N. Gu, C. Rao, Y. Tian, *et al.* Anti-inflammatory and antiapoptotic effects of mesenchymal stem cells transplantation in rat brain with cerebral ischemia. *J Stroke Cerebrovasc Dis* 2014;**23**(10):2598–2606.

138 K. S. Cho, M. K. Park, S. A. Kang, *et al.* Adipose-derived stem cells ameliorate allergic airway inflammation by inducing regulatory T cells in a mouse model of asthma. *Mediators Inflamm* 2014;**2014**:436476.

139 H. K. Lee, S. H. Lim, I. S. Chung, *et al.* Preclinical efficacy and mechanisms of mesenchymal stem cells in animal models of autoimmune diseases. *Immune Netw* 2014;**14**:81–88.

140 S. Hajizadeh-Sikaroodi, A. Hosseini, A. Fallah, *et al.* Lentiviral mediating genetic engineered mesenchymal stem cells for releasing IL-27 as a gene therapy approach for autoimmune diseases. *Cell J* 2014;**16**:255–262.

141 A. C. Bowles, B. A. Scruggs, B. A. Bunnell. Mesenchymal stem cell-based therapy in a mouse model of experimental autoimmune encephalomyelitis (EAE). *Methods Mol Biol* 2014;**1213**: 303–319.

142 C. Xie, D. Y. Xie, B. L. Lin, *et al.* Interferon-β gene-modified human bone marrow mesenchymal stem cells attenuate hepatocellular carcinoma through inhibiting AKT/FOXO3a pathway. *Br J Cancer* 2013;**109**:1198–1205.

143 D. Zhang, L. Zheng, H. Shi, *et al.* Suppression of peritoneal tumorigenesis by placenta-derived mesenchymal stem cells expressing endostatin on colorectal cancer. *Int J Med Sci* 2014;**11**: 870–879.

144 H. Kikuchi, H. Yagi, H. Hasegawa, *et al.* Therapeutic potential of transgenic mesenchymal stem cells engineered to mediate anti-high mobility group box 1 activity: targeting of colon cancer. *J Surg Res* 2014;**190**:134–143.

145 W. Ding, H. You, H. Dang, *et al.* Epithelial-to-mesenchymal transition of murine liver tumor cells promotes invasion. *Hepatology* 2010;**52**:945–953.

146 K. M. Akram, S. Samad, M. A. Spiteri, N. R. Forsyth. Mesenchymal stem cells promote alveolar epithelial cell wound repair *in vitro* through distinct migratory and paracrine mechanisms. *Respir Res* 2013;**14**:9.

147 X. Zhang, L. Zhang, W. Xu, *et al.* Experimental therapy for lung cancer: umbilical cord-derived mesenchymal stem cell-mediated interleukin-24 delivery. *Curr Cancer Drug Targets* 2013;**13**:92–102.

148 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**:e47559.

149 M. Duijvestein, A. C. Vos, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010;**59**: 1662–1669.

150 M. Abou-Shady, H. U. Baer, H. Friess, *et al.* Transforming growth factor betas and their signaling receptors in human hepatocellular carcinoma. *Am J Surg* 1999;**177**:209–215.

151 K. Chen, K. Man, H. J. Metselaar, *et al.* Rationale of personalized immunosuppressive medication for hepatocellular carcinoma patients after liver transplantation. *Liver Transpl* 2014;**20**:261–269.

152 R. Wieczorek-Godlewska, D. Miszewska-Szyszkowska, K. Slubowska, *et al.* Dramatic recurrence of cancer in a patient who underwent kidney transplantation – case report. *Transplant Proc* 2014;**46**: 2897–2902.

153 Q. Pan, S. M. Fouraschen, P. E. de Ruiter, *et al.* Detection of spontaneous tumorigenic transformation during culture expansion of human mesenchymal stromal cells. *Exp Biol Med (Maywood)* 2014;**239**:105–115.

154 L. Bindslev, M. Haack-Sørensen, K. Bisgaard, *et al.* Labelling of human mesenchymal stem cells with indium-111 for SPECT imaging: effect on cell proliferation and differentiation. *Eur J Nucl Med Mol Imaging* 2006;**33**:1171–1177.

155 D. L. Kraitchman, M. Tatsumi, W. D. Gilson, *et al.* Dynamic imaging of allogeneic mesenchymal stem cells trafficking to myocardial infarction. *Circulation* 2005;**112**:1451–1461.

156 M. El-Ansary, I. Abdel-Aziz, S. Mogawer, *et al.* Phase II trial: undifferentiated versus differentiated autologous mesenchymal stem cells transplantation in Egyptian patients with HCV induced liver cirrhosis. *Stem Cell Rev* 2012;**8**:972–981.

157 S. Terai, T. Ishikawa, K. Omori, *et al.* Improved liver function in patients with liver cirrhosis after autologous bone marrow cell infusion therapy. *Stem Cells* 2006;**24**:2292–2298.

158 A. Gholamrezanezhad, S. Mirpour, M. Bagheri, *et al.* *In vivo* tracking of $^{111}$In-oxine labeled mesenchymal stem cells following infusion in patients with advanced cirrhosis. *Nucl Med Biol* 2011;**38**:961–967.

CHAPTER 29

# Mesenchymal stem/stromal cell therapy: mechanism of action and host response

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## 29.1 Mesenchymal stem/stromal cells

Although the earliest identification of putative mesenchymal stem or stromal cells (MSCs) was described over 50 years ago by Alexander Friedenstein [1] closely following the emergence of the hematopoietic stem cell (HSC) field with the seminal work of Till and McCulloch in bone marrow [2], the MSC field has lagged behind somewhat in terms of defining the stem cell properties of differentiation, multipotentiality, and self-renewal. Friedenstein first isolated adherent fibroblastic cells from the stromal compartment of bone marrow. These cells were able to differentiate into osteocytes and establish colonies from a single cell *in vitro*, referred to as colony-forming units fibroblastic (CFU-F) [1,3,4]. More critically to defining their stem cell phenotype, the colony-forming cells were also capable of generating multiple skeletal tissues *in vivo* [5,6].

The earliest terminology describing these cells used either marrow stromal cells or mesenchymal stromal cells. Caplan first coined the phrase "mesenchymal stem cells" to associate the "stemness" shown by the capacity of the cells to form tissues of the mesenchymal lineage [7]. This has led to some confusion and controversy in the field, with Bianco and Robey using the term "skeletal stem cells" to describe the mesenchymal cell co-existing with HSCs in marrow [8]. These authors identified that human cells associated with a subendothelial location in marrow stroma and high surface expression of melanoma-associated cell adhesion molecule (MCAM or CD146) were capable of promoting the establishment of the hematopoietic microenvironment (HME) and self-renewing as identical subendothelial cells in an immunocompromised mouse *in vivo*. The cellular components of the bone marrow formed in the heterotopic bone ossicles were of host origin, but CD146-positive cells also formed bone, with human fibroblastic tissue, osteocytes, and osteoblasts identified in these chimeric ossicles [9]. Furthermore, the human $CD146^+$ adventitial reticular cells produced angiopoietin-1, a molecule associated with angiogenesis [9]. Another study defined the isolation and phenotype of murine skeletal stem cells. Additionally, these authors identified some progenitor populations involved in the differentiation pathways to bone, cartilage, and stromal tissue as well as potential regulators of lineage commitment [10].

Since the original work in human bone marrow, MSCs have been isolated and characterized from many other human sources and species. The isolated cells demonstrate the capacity to differentiate into bone, fat, cartilage, and other cells of connective tissue lineages *in vitro* [11,12]. The most commonly used sources other than marrow include adipose tissue [13–15], umbilical cord sources [16–19], and skeletal muscle [20]. However, MSCs

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have also been isolated from tissues such as synovium [21], articular cartilage [22,23], and ligament [24–27]. MSCs isolated from ligaments displayed similar properties to bone-marrow-derived MSCs but were also capable of *in vitro* differentiation to cells with characteristics of the ligaments themselves [25,28]. In a similar manner, MSCs isolated from synovium, perhaps the source of cartilage cells during development, are more chondrogenic than bone-marrow-derived cells or from MSCs isolated from joint-associated fat tissue [29,30]. Therefore, it is clear that MSCs seem to have the capacity to differentiate into cells of connective tissue lineages, such as cartilage, bone, and fat, *in vitro* whatever the tissue of origin but may have some tissue-specific characteristics.

Multipotential, pericyte-like cells with the capacity of *in vitro* differentiation to a number of lineages have also been isolated from blood vessels [31–35]. In addition to the work from Sachetti *et al.* described above, a number of other reports had suggested a perivascular niche as the source of MSCs derived from skeletal muscle, brain and adipose tissue [9,36–38]. This work, along with the wide tissue distribution of MSCs, led to the idea that MSCs derive from a perivascular niche [20] and a proposal from Caplan that although all MSCs are pericytes, not all pericytes are MSCs [39]. However, elegant work using a double-nucleoside-labeling strategy to examine distribution of stem cells before and after cartilage injury in the joint showed that MSCs in the joint were not pericytes. Proliferating, label-retaining cells after injury did not express hematopoietic and endothelial markers but showed a surface marker profile characteristic of MSCs [40].

Recent data looking at genome-wide methylation, transcription, and *in vivo* assessment of MSCs from various sources indicated that development potential differs depending on the cell source [41]. Despite very similar *in vitro* characteristics, only bone-marrow-derived MSCs, with a chondrogenic transcriptional program, were capable of establishing HME and forming bone. This report added to earlier data that regeneration of the mouse distal digit was mediated by local tissue-resident stem or progenitor cells that were lineage restricted [42]. Fracture repair is also associated with an MSC subpopulation from bone marrow: $Mx1^+$ cells exhibited a fate-restricted bone potential *in vivo* but underwent multilineage differentiation *in vitro* [43].

In addition to expanding knowledge on defining *in vivo* reparative cells it is also critical to establish the developmental origin of postnatal stem cells in order to advance the field of MSCs [10,44,45]. Mendez-Ferrer *et al.* have used the intermediate filament protein nestin to investigate the relationships between MSCs and HSCs in bone marrow [46]. This group recently showed that distinct biological functions ascribed to MSCs were associated with specific developmental origins and generated definitive data to indicate that not all MSCs are alike. MSCs negative for the neural marker nestin with an osteochondroprogenitor phenotype originated from mesoderm. However, nestin-positive cells derived from the neural crest contributed to the HSC niche in neonatal bone marrow [44] (and see also Chapter 32). This work added to an earlier study by Takashima *et al.* that showed a neural epithelium origin for early MSCs, some of which developed through a neural crest intermediate stage. These cells were replaced in early neonatal tissues [47]. Data on the developmental origin of MSCs is a significant advance in explaining the known heterogeneity of MSC preparations [48–50]. This heterogeneity in ancestral MSCs that may be present in source tissue is further compounded by the presence of multiple progenitor populations derived from the stem cells. *In vitro* culture could also add to this heterogeneity (see Chapter 7). This issue is highlighted by the reports on clonal heterogeneity of MSC preparations from many sources [51–55]. The use of MSCs for therapeutic applications may be compromised by this heterogeneity and the inability to specifically define the clinical population. It is also unknown whether the well-described clinical utility of MSCs is dependent on this heterogeneity or impacted negatively by the phenomenon.

Whatever the eventual outcome of research in this area, it is interesting to make the following speculations. HME-associated MSCs of neural origin may function as trophic mediators of repair, reflecting their capacity to provide a stromal support system for HSCs and to establish the HME in marrow [9,11,56,57]. These cells with a perivascular location will have widespread application in many tissue processes and provide the rationale for MSC-mediated effects in disease scenarios currently under investigation. Mesodermally derived MSCs or other tissue-specific stem cells will function to replace or regenerate damaged tissues. Furthermore, the perivascular MSC may act to recruit, activate, and direct differentiation.

## 29.2 Therapeutic application of mesenchymal stem/stromal cells

The use of MSC transplantation for tissue repair has received a great deal of attention in recent years, and these cells stand at the forefront of successful strategies in regenerative medicine [12,58–62]. Progress in preclinical models over the past decade and more recent evidence from clinical trials have led to some optimism that MSC therapy has the potential to be a transformative technology in medicine, although definitive and unambiguous proof of this is not yet forthcoming. Despite the surge of interest in this technology, there are very significant questions still unanswered, and several hurdles relating to biological understanding and translation that need to be overcome. While clinical proof of concept will emerge as trials are completed, technical deficits remain relating to both biology and logistics (Figure 29.1). In the biological context, three issues of great importance remain: potency, mechanism of action, and immune modulation. In logistical terms, issues of supply and delivery need to be addressed if the field is to develop unimpeded. Specifically, these include the incorporation of cell expansion protocols that include fully xeno-free growth media, bioreactors capable of industrial-scale cell manufacturing, and

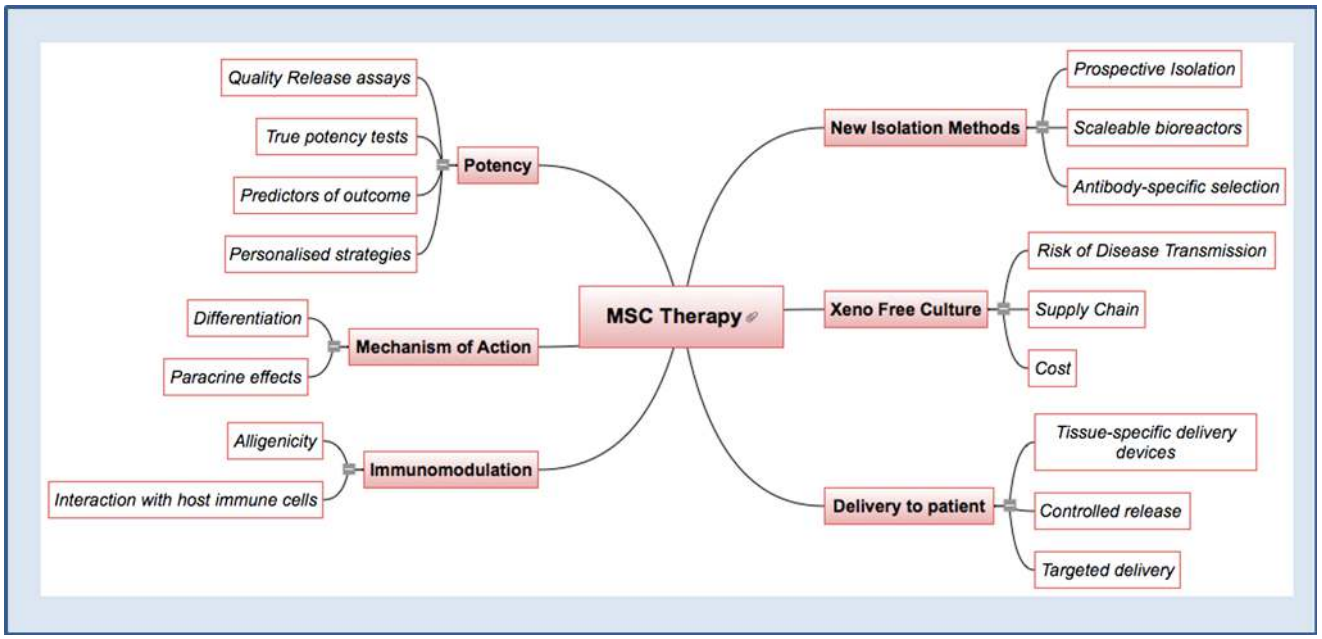



**Figure 29.1** MSC therapy: obstacles to translation. The translation of MSC therapy requires further development in the areas of isolation methods, xeno-free culture, potency assessment, mechanisms of action, immune modulation, and delivery to the patient.

tissue-specific device technologies for delivery of cell doses to patients [58,61,63].

The concept of potency is widely misapplied in the field of MSC therapy. The term is used in pharmacology to refer to the amount of the therapeutic agent needed to produce a specific measured outcome. It implies an understanding of both dose and mechanism, neither of which is available in the case of MSC therapy. Currently used markers of cell potency, such as those proposed by the International Society for Cell Therapy, are of limited value because they lack cell specificity and relationship to clinical outcome. In the context of quality control, product release testing, and dose adjustment, potency will need to be rigorously and fully addressed.

A complete understanding of mechanism of action is needed in order to optimize cell dose and develop next-generation products, including disease-specific and personalized therapies. This understanding will only emerge if the activity of cells *in situ* is understood; that is, the molecular and cellular impact that transplanted MSCs have on the host within the injury niche. This impact will relate possibly to differentiation of the delivered cells to the local phenotype and their structural contribution to tissue repair. It may also relate to the paracrine effects that the transplanted cells have, mediated through one or many secreted factors that evoke a host response that leads to a repair outcome. It is not likely that a complete understanding of mechanism of action will come from studying cells *in vitro* but will require instead probing the phenotype of cells delivered to the injured host. This will require sophisticated experiments in cell tracking and retrieval.

The interaction between transplanted cells and cells of the host immune system needs to be fully understood. It is also necessary to fully understand allogenicity – the use of mismatched donor rather than patient-derived (autologous) cells. This is likely to represent a more useful commercial manufacturing model and will make product more widely available for rapid use. Although allogeneic MSCs are widely used in preclinical and clinical applications, there are aspects of allogenicity that require greater understanding. These include, among others, a fuller understanding of the impact of delivered allogeneic cells on the host immune system, whether these effects change if the cells differentiate, and what are the consequences of the presence of alloantibodies in the MSC recipient in terms of future transplants.

In the logistical area, steps are urgently needed to guarantee the consistency, quality, and safety of MSC products. There is a need for serum-free and animal-protein-free culture systems that will remove the dependence on fetal bovine serum. This is an issue of safety relating to the risk of disease transmission associated with products of bovine origin, and one of supply chain relating to limited availability of these materials. In addition, there is a need for new and efficient isolation methods that are biologically specific and more reproducible. There is a risk associated with plastic adherence as a method for isolating MSCs from tissues. This is associated with the heterogeneity of cell populations isolated in this way, and a more defined method that relies on specific biological characteristics of the cells may be more desirable as mentioned earlier. Such a method might involve prospective isolation using antibody selection as

an example. Lastly, but by no means least, there is a need now to focus on delivery devices for cell therapy. This has received little attention to date, but it is unlikely that cell transplantation will be widely used unless there are target-specific, controlled devices for cell delivery. These are likely to be sterile, single-use closed systems that will allow cryopreserved MSC-to-patient delivery with minimal or no manipulation of the cells at the clinical site and thus minimal risk of contamination and operator error.

The interaction between the transplanted cells and the host is critical and is addressed in this chapter. This critical aspect of the therapeutic effect of MSCs is considered both from the point of view of tissue repair and immune modulation.

## 29.3 Mechanism of action

As mentioned earlier, MSCs were first isolated from bone marrow by Friedenstein and described as adherent, colony-forming fibroblastic cells with bone-forming activity [1,64]. The notion that MSCs could be delivered to injured tissues to effect a repair process was first proposed by Caplan [7]. Early efforts focused on conditions that were related to the mesenchymal nature of the cells and included bone repair [65–69], tendon repair [70,71], cartilage resurfacing [72,73], spine fusion [74–76], treatment of osteogenesis imperfecta [77–80], and osteoarthritis [81]. In recent years that focus has shifted to many other conditions, including vascular disease, myocardial infarction, diabetes, Crohn's disease, graft-versus-host disease (GvHD), spinal cord injury, and stroke. Currently, approximately 400 clinical trials are listed on the clinical trials website of the US National Institutes of Health (Clinicaltrials.gov).

There are two major paradigms of tissue regeneration associated with MSC transplantation. The first, which is the founding premise of cellular therapy, states that MSCs act as multipotent competent cells capable of migration, engraftment, and differentiation to the appropriate lineage, and that the newly formed tissue is populated by transplanted cells. This was the basis of many earlier efforts in cardiac repair, where it was assumed that differentiation of the transplanted cells to a cardiomyocyte phenotype was the central element of repair [82]. It is also presumed to be an element in MSC-mediated cartilage resurfacing [72]. However, in the earliest report describing protection by MSCs from development of osteoarthritis after meniscectomy, no implanted cells were detected in the cartilage. Cells were detected at the surface of regenerated menisci; however, the vast majority of cells in the neo-tissue were host derived [81]. This differentiation paradigm has been added to, or replaced by, a second paradigm involving trophic mechanisms where the transplanted cells secrete immunomodulatory and repair factors that stimulate a host repair response [83]. This concept suggests that MSCs have the capacity to recognize and respond to the injured or inflamed environment by becoming activated, and several mechanism have been proposed that underpin this concept. The undeniable consequence of this is that the phenotype of culture-expanded MSCs measured *in vitro* may bear little relationship to their activity *in vivo*.

Many efforts have been made to dissect the molecular pathways and signals that are activated by transplanted MSCs. This effort has identified several putative candidates that may mediate the repair effects. Little has been done, however, to validate these *in vivo*.

One approach has led to the identification of the anti-inflammatory protein, tumor necrosis factor (TNF)-α-stimulated gene 6 protein (TSG-6). When human MSCs were delivered to infarcted rat hearts the transplanted cells showed high levels of expression of this gene [84]. In these experiments, the effect of delivery of MSCs was a decrease in matrix metalloproteinase activity and decreased infiltration of monocytes and granulocytes in the infarcted hearts. These effects were reduced when MSCs were treated with small interfering RNA to block TSG-6 expression. Also, when recombinant TSG6 was delivered to the hearts the therapeutic effects of MSCs were recapitulated. This work suggested that TSG-6 is a major mediator of the anti-inflammatory effects of MSCs. Further, in zymosan-induced peritonitis in mice it has been suggested that delivered MSCs are activated by the inflammatory environment to produce TSG-6, which then exerts an effect on macrophages and other cells to reduce this inflammatory cascade. Thus, the therapeutic mechanism depicted in these experiments involves a bidirectional signaling mechanism whereby MSCs transplanted to the injured or inflamed environment are activated or "licensed" to increase production of TSG-6, which then binds CD44 on macrophages and causes a decrease in nuclear translocation of nuclear factor kappa light chain-enhancer of activated B cells (NF-κB).

TSG-6 is an extracellular hyaluronan-binding protein, likely to have roles in cell–matrix interactions [85]. It is also a C–X–C motif chemokine ligand (CXCL)8-binding protein and suppresses CXCL8-mediated chemotaxis of neutrophils [86]. Furthermore, in the mouse model of zymosan-induced peritonitis, TSG-6 binds the CD44 receptor on resident macrophages to decrease the zymosan/toll-like receptor (TLR)2-mediated nuclear translocation of NF-κB [87]. The consequence of this may be profound in terms of attenuating the inflammatory response.

A number of other paracrine factors have been associated with MSC-mediated repair. For example, MSCs overexpressing Akt showed dramatic improvement in mediating a positive repair response in infarcted myocardium [88]. Factors such as vascular endothelial growth factor (VEGF), fibroblast-derived growth factor 2, hepatocyte growth factor (HGF), and insulin-like growth factor-1 (IGF-1) were implicated in this effect, and further work focused on secreted frizzled related protein 2, which acts to modulate Wnt signaling, increase cellular β-catenin and upregulates expression of antiapoptotic genes [89,90]. Thrombospondin (TSP)-2, described in 2002 as a secreted factor involved in autocrine regulation of MSC growth [91], was subsequently implicated in mediating cartilage repair in a rabbit

osteochondral model. TSP-2 was selected from an antibody array analysis of human umbilical-cord-derived MSC conditioned media (CM) collected after the cells were exposed to human osteoarthritic synovial fluid. Recombinant TSP-2 was able to improve *in vivo* cartilage repair, although not to the same extent as the umbilical-cord-derived MSCs themselves, with mechanistic insights suggesting the stimulation of endogenous chondroprogenitor cells [92]. Secreted factors from stromal cells isolated from adipose tissue have also been shown to impact hypertrophy and dedifferentiation of human chondrocytes *in vitro*, an effect that was partially mediated by hepatocyte growth factor [93]. In an *in vivo* study, mouse stromal cells isolated from adipose tissue activated by the inflammatory osteoarthritic environment were also shown to be chondroprotective, a phenomenon associated with a decrease in the calcium binding protein S100A8/A9 alarmin levels [94]. (The term "alarmin" has been proposed to categorize endogenous molecules that signal tissue and cell damage. Effector cells of both innate and adaptive immunity can secrete alarmins when they are activated by pathogen-associated molecular patterns or other alarmins. Endogenous alarmins and exogenous pathogen-associated molecular patterns therefore convey a similar message and elicit similar responses. They can be considered subgroups of a larger set, the damage-associated molecular patterns.)

Further insight into potential mechanisms of MSC-mediated repair has come from studies of cell-derived exosomes, which are nanovesicles derived from cell membranes. These secreted vesicles have a phospholipid bilayer membrane rich in cholesterol and are capable of transferring proteins, mRNA, and microRNA to adjacent cells. They are widely understood to be important for intercellular communication. They carry membrane-bound proteins that mediate interactions with ligands on other cells and matrix molecules. Examples include the transferrin receptor (CD9), tumor necrosis factor receptors, lactadherin, integrins, and tetraspanin (CD63, CD81) proteins. These membrane-bound molecules provide a potential mechanism for the homing of exosomes to a specific tissue or microenvironment.

One study evaluated the effects of MSC CM on repair of myocardial ischemia in pigs. Both intravenous and intracoronary delivery of the CM resulted in a 60% reduction in infarct size and marked improvement of systolic and diastolic cardiac performance. Fractionation of the CM indicated that the active component was an exosomal fraction complex of 50–200 nm in diameter. Electron microscopy showed that these complexes were phospholipid vesicles. They contained co-immunoprecipitating exosome-associated proteins CD81, CD9, and ALIX (also known as programmed cell death 6-interacting protein) and could be purified with a hydrodynamic radius of 55–65 nm by size exclusion fractionation using high-performance liquid chromatography. Purified exosomes administered in a mouse myocardial ischemia–reperfusion injury model revealed that MSCs mediate their cardioprotective paracrine effect by exosome secretion [47]. These results suggested that exosomes are a highly efficacious therapeutic agent that neutralizes myocardial ischemia–reperfusion injury and will likely be an effective adjuvant to complement current reperfusion therapy.

## 29.4 Host immune response to autologous mesenchymal stem/stromal cells transplantation

Following the initial identification of MSCs from the bone marrow [95], studies focused on the regenerative capacity of MSCs based on their potential to transdifferentiate to cells of mesenchymal lineages [55,81,96]. However, the discovery of the immunomodulatory potential of MSCs just over a decade ago revolutionized interest in MSCs as a cellular therapy for tissue regeneration [97,98]. Understanding the mechanisms by which MSCs modulate an inflammatory microenvironment has been the focus of intense research since then [99]. It has become apparent that MSCs can exert effects on cells of both the innate and adaptive immune systems [99] through cell-contact, cytokines, chemokines, and TLR-mediated mechanisms (see Chapters 33 and 34). MSCs can suppress the activation and function of various immune system cells, including macrophages, natural killer (NK) cells, T cells, and B cells [100–103]. These distinctive regenerative and immunomodulatory characteristics have seen their application in a wide variety of diseases, including GVHD [104–106] (see Chapter 57), cardiovascular diseases [107] (see Chapters 45 and 46), neurological diseases [108–110] (see Chapters 48 and 49), organ transplantation [111,112] (see Chapter 56), experimental autoimmune encephalomyelitis (EAE) [108–110], autoimmune diseases (see Chapter 54), rheumatoid arthritis [113], osteoarthritis [81], bone and cartilage defects [114] (and see Chapter 47), inflammatory bowel disease [115–117] (see Chapter 50), inflammatory lung disease [118] (Chapter 52), and allergic airway disease [119] (see Chapter 39).

## 29.5 Mesenchymal stromal cells in an inflammatory microenvironment

The ability of MSCs to home to sites of inflammation and adopt a distinct phenotype in response to the inflammatory microenvironment is crucial to our understanding of their therapeutic potential in acute and immune-mediated diseases. MSCs migrate to sites of tissue injury and suppress inflammation, which enables tissue-resident stromal cells to proliferate, modulate inflammation, support angiogenesis, and reconstruct the extracellular matrix, thus enabling wound healing [120–122] (Figure 29.2).

Additionally, an emerging concept is that MSCs become "licensed" or "activated" by exposure to inflammatory cytokines in the microenvironment, further emphasizing their distinctive phenotype [99]. Activation of MSCs by the inflammatory cytokines interferon (IFN)-γ, TNF-α, and IL-1β has been shown to enhance their ability to modulate immune responses [123].

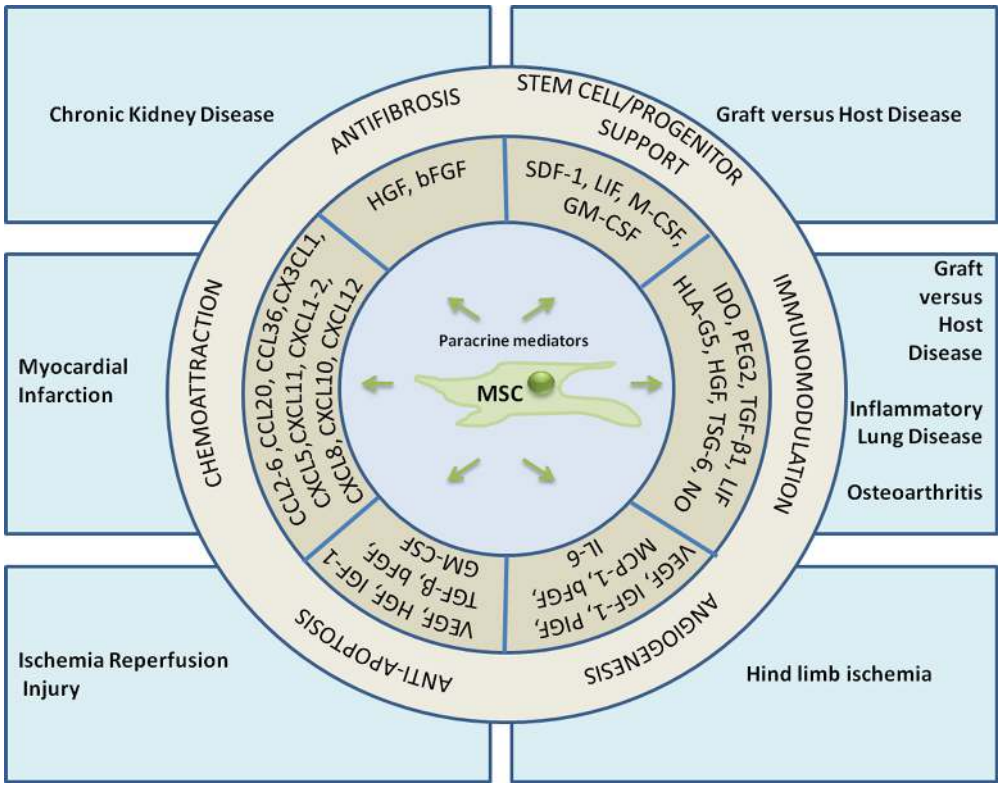



**Figure 29.2** MSC paracrine-mediated mechanisms of action and their therapeutic relevance. The therapeutic effects of MSCs are mediated in a large part through the release of paracrine factors. MSCs, as a result of their mechanisms of action, have potential in the treatment of diseases such as GvHD, inflammatory lung disease, osteoarthritis, hind limb ischemia, ischemia–reperfusion injury, myocardial infarction, and chronic kidney disease (outer boxes). Mechanisms of potential therapeutic relevance include the ability of MSCs to support stem cell/progenitor cells, immune modulation, angiogenesis, antiapoptosis, antifibrosis, and chemoattraction (indicated in the outer circle). MSCs can stimulate mitosis of tissue intrinsic progenitor or stem cells by secretion of stem cell factor, macrophage colony-stimulating factor (M-CSF), stromal cell-derived factor-1 (SDF-1, also known as CXCL12), leukemia inhibitory factor (LIF), and angiopoietin 1. The immunomodulatory effects of MSCs are mediated by indoleamine 2,3-dioxygenase (IDO), prostaglandin $E_2$ ($PGE_2$), transforming growth factor (TGF)-β1, LIF, human leukocyte antigen G5 (HLA-G5), HGF, TSG-6, and nitrous oxide (NO). MSCs can stimulate local angiogenesis by secretion of extracellular matrix, VEGF, IGF-1, placenta growth factor (PLGF), monocyte chemoattractant protein 1 (MCP-1, also known as CCL2), basic fibroblast growth factor (bFGF), and interleukin (IL)-6. MSCs have been shown to prevent apoptosis through HGF, TGF-β, IGF-1, bFGF, VEGF, and granulocyte M-CSF (GM-CSF). MSCs can elicit leukocyte and monocyte migration to sites of tissue injury to restore normal tissue homeostasis. Lastly, MSCs contribute to inhibition of scarring through the release of soluble factors, including HGF and bFGF.

Interestingly, activated MSCs have an enhanced homing potential and tissue regeneration properties [116,124]. This enhanced immunosuppressive ability has been attributed to the differential regulation of a number of key molecules, including IDO, NO, $PGE_2$, TGF-β, and TSG-6 by MSCs following proinflammatory stimulation. Other factors besides inflammatory cytokines also participate in "licensing" the immunomodulatory ability of MSCs [101]. Microbe-associated molecular patterns can activate MSCs through TLRs such as TLR3 and TLR4 [102,125]. TLR signaling has been shown to alter MSC-mediated immune modulation. TLR3 and TLR4 ligation may differentially induce MSCs to anti- and proinflammatory phenotypes respectively [125]. The emerging concepts that MSCs can sense their microenvironment and become differentially polarized depending on the nature and intensity of the inflammatory signals present adds another layer of complexity to our understanding of how the local microenvironment influences MSC activation and MSC-mediated immune modulatory functions [99,101,126] (Figure 29.3).

The nature of the inflammatory tissue microenvironment consequently influences and determines the fate of administered MSCs and may result in different therapeutic outcomes [123,127]. A complete understanding of MSC response to different phases and intensities of inflammation is necessary to guide strategies that will enable precise manipulation of therapeutic outcomes. Knowledge of how to control and regulate the immunomodulatory effects in variable inflammatory environments, both endogenously and exogenously, may provide an important new modality for improved clinical application.

MSC modulation of the immune system occurs as a multistage process involving MSC migration to the site of injury and response to inflammation, their response and activation by

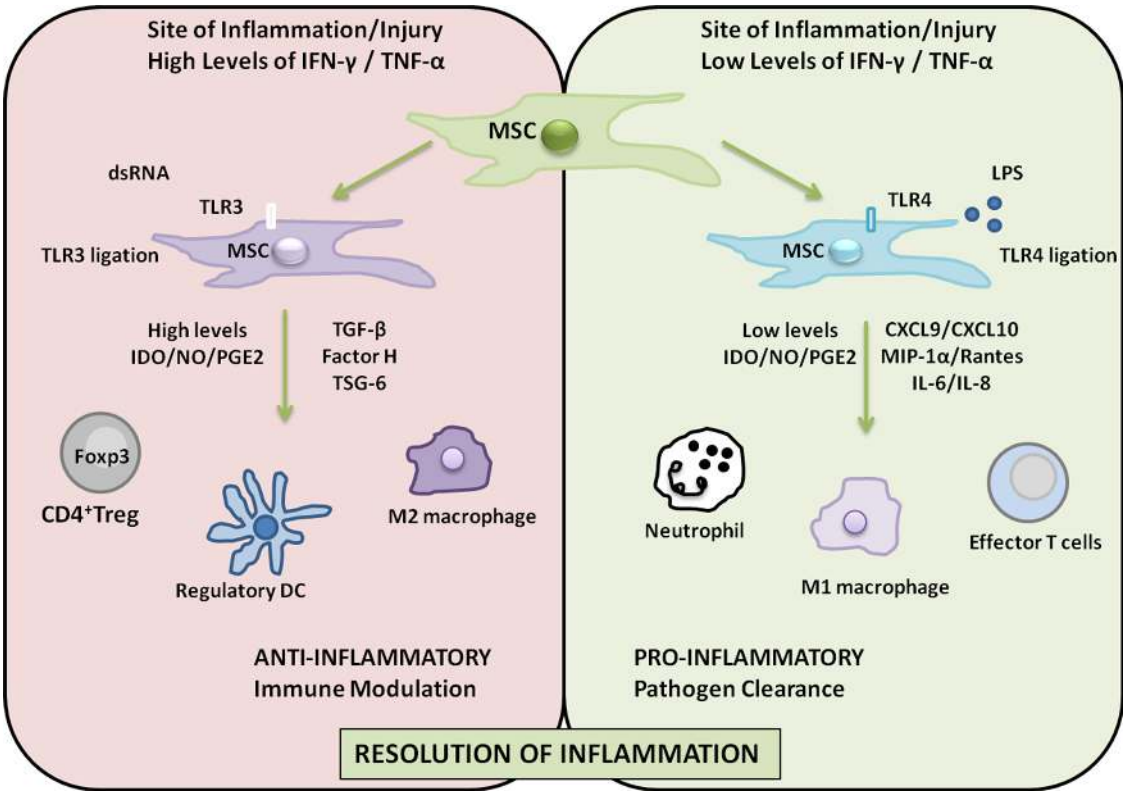



**Figure 29.3** MSC responses to diverse inflammatory microenvironments. Proinflammatory cytokines such as IFN-γ, TNF-α, or IL-1β are present in high concentrations at sites of acute or relapsing phases of inflammatory diseases. In response to high concentrations of these cytokines, and/or following ligation of TLR3 on MSCs, they produce large amounts of NO, IDO, and $PGE_2$, which participate in MSC-mediated immune modulation by induction of regulatory T cells (Tregs), regulatory dendritic cells (DCs), and M2 macrophages (left panel). In the context of chronic inflammation, in the presence of low concentration of proinflammatory cytokines and/or TLR4 ligation, MSCs secrete low levels of NO, $PGE_2$, and IDO, as well as CXCL9/10, IL-8, and IL-6. MSCs may promote pathogen clearance in this context by polarization of proinflammatory M1 macrophages, antimicrobial activity, and through promotion of the survival of neutrophils. This differential ability to respond to inflammation enables MSCs to promote pathogen clearance followed by suppression of an immune response in the resolution of inflammation. LPS: lipopolysaccharide; MIP-1α: macrophage inflammatory protein 1α.

inflammatory mediators, promotion of pathogen clearance, and subsequent modulation of inflammation [99]. MSCs exert effects that are both contact dependent and independent and influence cells of both the innate and adaptive immune systems (Figure 29.4).

## 29.6 Mesenchymal stem/stromal cells-mediated immunomodulation of the innate immune system

Innate immune cells have important roles in tissue homeostasis and are the first line of defense against invading pathogens. MSC and innate immune cell interactions shape inflammatory and regenerative environments [103]. Monocytes, macrophages, DCs, neutrophils, mast cells, and NK cells are all components of the innate immune system. MSCs can modulate the effector functions of each of these innate cellular subsets, which have been reviewed in great detail previously [99–103,121,122,128,129].

Macrophages have key roles at sites of inflammation and can be polarized by the microenvironment into classically activated M1 macrophages, which are proinflammatory and involved in antimicrobial activity, or alternatively activated M2 macrophages, which are involved in tissue repair and resolution of inflammation [103]. MSCs recruit monocytes and macrophages into inflamed tissue in a CCL3-, CXCL2-, and CCL12-dependent manner. MSCs induce M2 macrophage polarization. M2 macrophages have high levels of CD206, IL-10, and phagocytic activity and low levels of TNF-α and IFN-γ. (CD206, also known as mannose receptor, is a pattern recognition receptor belonging to the C-type lectin superfamily. It is expressed on macrophages, DCs, Langerhans cells, and hepatic or lymphatic endothelial cells.)

These effects have been shown to be mediated by both cell-to-cell contact (EP2/4 ($PGE_2$ receptors), CD44, TLR2 on macrophages) and soluble factor ($PGE_2$, TGF-β, and TSG-6) mechanisms [87,130]. Macrophage-secreted TNF-α can activate MSCs to secrete TSG-6, thereby inhibiting inflammation through CD44-receptor-mediated negative feedback on macrophages [87]. The relevance of MSC-mediated modulation of macrophages has been validated in various models of sepsis and zymosan-induced peritonitis, in which MSCs promoted

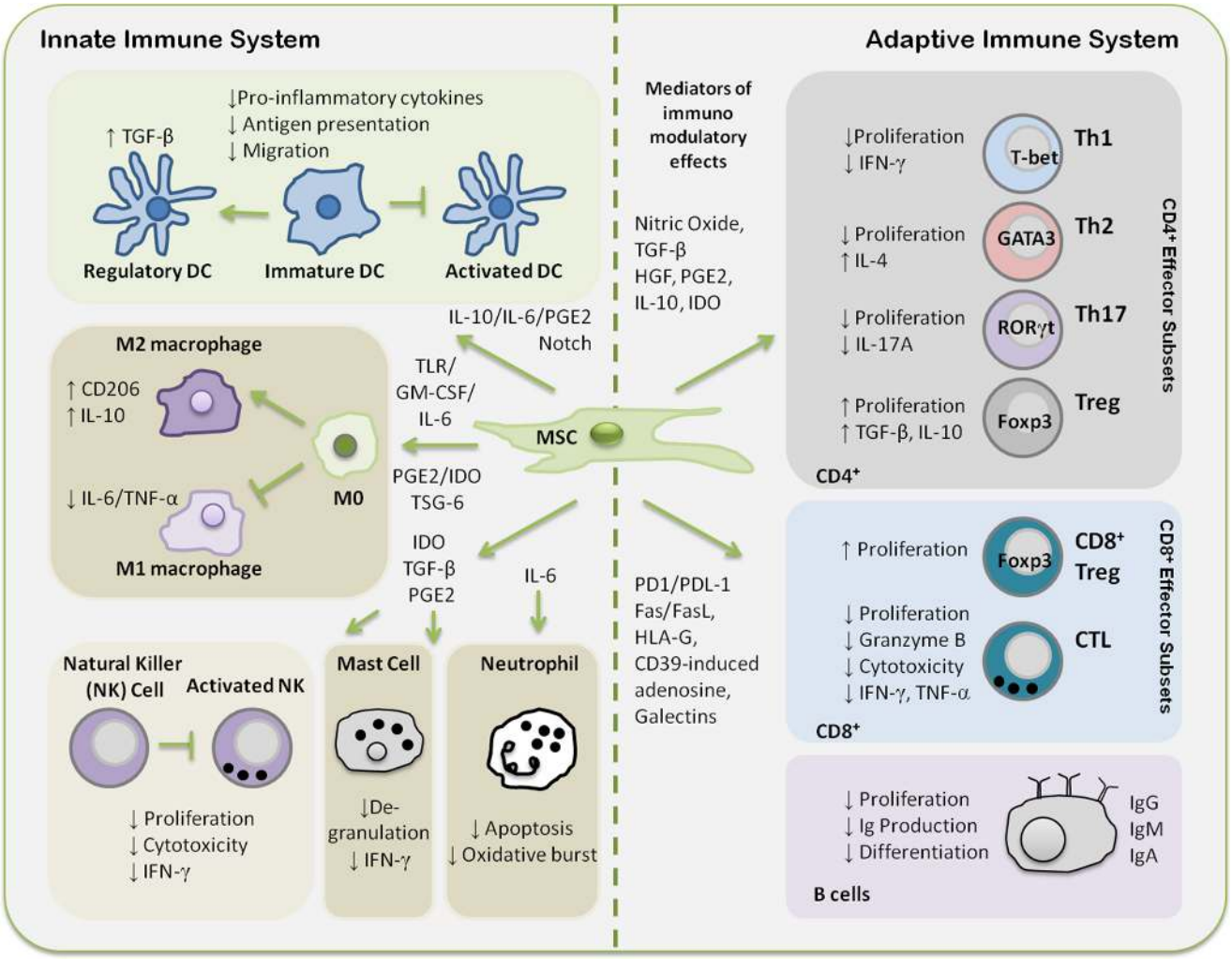



**Figure 29.4** MSC-mediated mechanisms of immune modulation. A summary of MSC-mediated effects on components of the innate and adaptive immune system is shown. These effects are mediated by soluble and cell-surface protein interactions. MSCs can modulate the effector functions of innate cellular subsets. MSCs inhibit DC maturation, migration, and antigen presentation ability, as well as promotion of regulatory DC subsets. MSCs promote the differentiation of monocytes to an anti-inflammatory M2 macrophage phenotype, while inhibiting the development of M1 proinflammatory macrophages. MSCs inhibit NK cell activation and cytotoxicity, mast cell degranulation, and neutrophil apoptosis and oxidative burst. MSC secretion of IL-6, IL-10, $PGE_2$, TGF-β, TSG-6, and GM-CSF, as well as Notch and TLR signaling, have been shown to modulate these MSC-mediated effects on innate immune cell responses. In the context of the adaptive immune system, MSCs inhibit the proliferation of T cell effector subsets, including $CD4^+$TH1, TH2, and TH17 effectors and can promote the expansion of Tregs in an antigen-specific manner. MSCs inhibit the activation and proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs) but induce the expansion of $CD8^+$ Tregs. MSCs can inhibit B cell proliferation and suppress the production of immunoglobulin (Ig)G, IgA, and IgM *in vitro*. Programmed cell death protein 1 (PD1)/programmed death ligand 1 (PDL-1), Fas/FasL interactions, as well as MSC secretion of NO, TGF-β, IDO, HGF, $PGE_2$, and IL-10 have been shown to modulate these MSC-mediated effects on adaptive immune cell responses. (See color plate section.)

resolution of inflammation that was dependent on monocyte/macrophage recruitment [87,130]. In the context of chronic inflammation and in the presence of low concentrations of proinflammatory cytokines and/or TLR4 ligation, MSCs secrete low levels of NO, $PGE_2$, and IDO, as well as CXCL9/10, IL-8, and IL-6. MSCs may promote pathogen clearance in such a microenvironment by polarization of antimicrobial activity [99] by proinflammatory M1 macrophages and through promotion of neutrophil survival. Neutrophils, another key facilitator of antimicrobial responses, produce a large quantity of microbicidal oxidative products in response to the detection of microbial molecules in the so-called "respiratory burst." During early-stage inflammation the proinflammatory activities of MSCs can facilitate neutrophil recruitment, accumulation, and phagocytosis to enable pathogen clearance, which is IL-6, IFN-β, and GM-CSF dependent [102]. MSCs inhibit neutrophil apoptosis even under IL-8-mediated activation conditions via MSC-derived IL-6 [131]. MSCs also prevent respiratory bursts from neutrophils,

although chemotaxis is unaffected. MSCs also inhibit mast cell functions, including migration, TNF production, and IgE-cross-linking-mediated degranulation, in a $PGE_2$-dependent-manner [132,133]. These findings are particularly interesting in the context of allergy and autoimmunity, in which MSC administration has been therapeutically efficacious. NK cells have a critical role in eliminating intracellular pathogen-infected cells, stressed cells, and cancer cells. NK cytotoxicity is regulated by both inhibitory and activating receptors, as well as by target cell major histocompatibility complex (MHC) expression levels and antibody-dependent cellular cytotoxicity. MSCs inhibit NK cell proliferation and suppress the expression of activating receptors in a $PGE_2$-, IDO-, and TGF-β-dependent manner, although these effects are context dependent [121]. DCs bridge the innate and adaptive immune systems and have a critical role in host defense and generation of immune responses. MSCs inhibit maturation of DCs by contact-dependent and -independent mechanisms [134–136]. MSCs have been shown to influence key features of DC function both *in vitro* [134] and *in vivo* [137], including migration, maturation, and antigen presentation [134,137]. MSCs can also reprogram conventional lymphocyte stimulatory DCs into anti-inflammatory DCs with a tolerogenic phenotype [138], which has implications in the context of solid organ transplantation (Chapter 56). In fact, tolerogenic DCs induced by MSCs inhibit delayed-type hypersensitivity responses, prevent $CD4^+$ T cell activation, and promote the survival of cardiac allografts [137,139,140] (Figure 29.3). Emerging evidence demonstrates that MSCs modulate the function of innate immune cells to establish a fine balance between pathogen elimination, resolution of inflammation, and tissue repair.

## 29.7 Mesenchymal stem/stromal cells-mediated immune modulation of the adaptive immune system

The adaptive immune system functions in an antigen-specific manner and is critical to the development of immunological memory [102]. The adaptive immune system is composed of $CD4^+$ helper T lymphocytes and $CD8^+$ CTLs that facilitate an antigen-specific immune response following presentation of antigen by antigen-presenting cells. It is now well documented that MSCs have potent immunomodulatory effects on most cellular subsets of the adaptive immune system, including $CD4^+$ TH1, TH2, and TH17 cells, CD8 CTLs, $CD4^+$ Tregs, $CD8^+$ Tregs, and B cells, and these mechanisms have been reviewed in detail previously [99–103,121,122,128,129,141]. MSCs suppress $CD4^+$ helper T cell proliferation (TH1/TH2/TH17) *in vitro* by contact-dependent (Fas/FasL, PD1/PDL1) and -independent mechanisms (TGF-β, HGF, $PGE_2$, IDO, and NO), which can be enhanced by MSC activation with proinflammatory cytokines [126,141]. This inhibition occurs as a result of cell cycle phase arrest in $G_0/G_1$ [99], regardless of the type of stimulation. Although it is evident that MSCs can suppress T cell proliferation and activation directly, it has recently been suggested that this suppression is dependent on the presence of monocytes [142]. MSCs can suppress activation and cytotoxicity of CTLs [141]. Several studies have shown that MSCs can polarize T cells toward a regulatory phenotype that represents another mechanism by which MSCs can suppress inflammation. Tregs are a subpopulation of helper T cells that are characterized by the expression of the forkhead box P3 (Foxp3) transcription factor and are specialized in suppressing T-cell-mediated immune responses. The generation of MSC-induced Tregs is both contact dependent (with helper T cells) and independent (through $PGE_2$, TGF-β, and HLA-G5) [102,143], and has also been shown to be dependent on the presence of monocytes [144,145]. Additionally, studies have shown that depletion of Tregs abrogates the anti-inflammatory effect mediated by MSCs [139,146]. Moreover, MSC-mediated induction of $CD8^+$ Tregs has been demonstrated to be immunosuppressive in human allo-antigen-stimulated cultures [141]. Treg expansion has been associated with protection in a number of disease models, including allergic airway disease [147] and inflammatory bowel disease [148]. MSCs also suppress B cell proliferation, chemokine receptor expression, and the production of IgG, IgM, and IgA *in vitro*. In transplantation studies, MSCs suppressed allo-specific antibodies and promoted long-term graft acceptance [139]. These effects are both contact dependent through the PD1/PDL1 pathway and independent. Together, these mechanisms clearly demonstrate immense potential for the use of MSCs in modulating the host adaptive immune response, particularly in the context of transplantation and allergic, inflammatory, and autoimmune diseases.

## 29.8 Host immune response to transplantation of allogeneic mesenchymal stem/stromal cells

Identification of the potent immunosuppressive properties of MSCs has led to the broadly accepted principle that MSCs from genetically unrelated individuals (allogeneic MSCs) could be used therapeutically with equal efficacy to autologous MSCs and without triggering a specific response against the donor MSCs typically associated with allogeneic organ transplants [149,150]. While the use of autologous MSCs may be thought more desirable from the perspective of safety of administration and avoiding the possibility of an allogeneic immune response, certain clinical situations, in which autologous MSCs are unattainable, dysfunctional, and/or less effective owing to disease [151–153], require the availability of "off-the-shelf" allogeneic MSCs. As a result, allogeneic MSCs from young healthy donors are an attractive source of cells for such clinical situations.

A range of studies have investigated the efficacy of allogeneic MSCs in preclinical disease models [154–165], both with and without direct comparison to autologous MSCs [149]. These

studies have provided insight into the range of antidonor responses that may be encountered in various clinical applications for allogeneic MSCs [149] and which have increased dramatically over the past several years [149,166–169]. Allogeneic MSC immunogenicity and the functional nature of antidonor immune responses generated following the administration of allogeneic MSCs have been determined using many approaches, including *in vivo* persistence of allogeneic MSCs after their administration, immune cell infiltration, therapeutic efficacy, analysis of antidonor T cell and B cell responsiveness, measurement of serum alloantibodies, *in vitro* "recall" of T cell responses to donor-specific alloantigens, and *in vivo* rechallenge experiments involving readministration of cells from the same or a different donor. Griffin *et al.* reviewed numerous experimental studies showing that allogeneic MSCs trigger donor-specific cellular and humoral immune responses [149]. These findings bring into question whether allogeneic MSCs are as immune privileged as initially hoped and whether antidonor immune responses limit their efficacy. Various preclinical and clinical studies provide evidence for a therapeutic benefit of allogeneic MSCs. However, the anti-inflammatory and immune modulatory properties of MSCs do not prevent the generation of active donor-specific T and B cell responses against allogeneic MSCs *in vivo*. The consequences of these observations have not been fully investigated. It is worth noting that allogeneic MSCs have been administered in human trials by multiple routes without severe immunological complications. However, large-scale therapeutic application of allogeneic MSCs will require a broader understanding of the nature and future consequences of the resulting antidonor responses. It is necessary to consider whether alloantibodies or memory T cells generated following the administration of allogeneic MSCs will represent a significant barrier to their successful administration [149,169]. A better understanding of the underlying mechanisms of the immune responses generated in response to the administration of allogeneic MSCs may be useful to either prevent or modify them in order to optimize clinical benefit.

There is a growing interest in the use of "activated" or "licensed" allogeneic MSCs based on the premise that MSC immunosuppressive properties are enhanced by pretreatment with inflammatory mediators such as IFN-γ [128,170] and TNF-α [126]. However, in the context of allogeneic MSCs, the increased anti-inflammatory potency of IFN-γ-activated MSCs may be counterbalanced *in vivo* by increased immunogenicity. Several studies have shown upregulation of key surface proteins, including MHC class I antigens, MHC class II antigens, intracellular cell adhesion molecule, and costimulatory molecules following activation of allogeneic MSCs with IFN-γ [149]. This increased immunogenicity was confirmed by observations of enhanced T cell responses and production of donor-specific antibodies, which resulted in accelerated clearance of a second injection of allogeneic MSCs *in vivo* [171]. Nevertheless, enhanced therapeutic efficacy has been observed with activated allogeneic MSCs in a model of GVHD [172]. These findings highlight the need to better understand how the potential for increased efficacy and enhanced immunogenicity can be balanced in the context of inflammatory disease. It has been suggested that protecting MSCs from immune detection and prolonging their persistence *in vivo* may improve clinical outcomes and prevent patient sensitization toward donor antigens [173].

The immunogenicity of MSCs following differentiation to chondrocytes, osteocytes, or other lineages is also poorly characterized and may be of fundamental importance for cell therapy for myocardial, bone, or joint disease [174,175]. Even weak immunogenicity may hamper successful translation for clinical applications that may require MSC engraftment, such as myocardial infarction (but see Chapter 45) or osteoarthritis. Recent evidence suggests that MSCs become more immunogenic and lose their immunosuppressive properties after differentiation [107,174–176]. Heightened immune responses have been confirmed *in vivo* [107,175,176]. Studies have shown that lack of immunosuppression was associated with a loss of $PGE_2$ and that immunosuppressive ability could be reestablished with addition of exogenous $PGE_2$ [176]. These findings raise important questions regarding the immunogenic properties of allogeneic MSCs following *ex vivo* or *in vivo* differentiation that are likely to become more clinically relevant as cellular therapies become established for specific acute and chronic diseases. It is necessary to determine if allogeneic immune responses limit therapeutic efficacy or long-term survival of MSCs *in vivo*, or whether these limitations can be overcome by genetic modification or the use of immunosuppression agents after administration of allogeneic MSCs.

## 29.9 Summary

It is becoming increasingly apparent that MSCs, although initially thought to be potent suppressors of immune cells, are distinctively responsive to crosstalk within the host microenvironment. Exploiting the potential of MSCs will involve a thorough understanding of the crosstalk between MSCs and endogenous immune cells in both physiological and pathological situations. Our current level of understanding of the host response with regard to allogeneic MSCs, although incomplete, suggests that the concept of immune privilege should be reevaluated. Emerging evidence indicates that MSC-associated alloantigens are actively recognized by the recipient's immune system with a range of potential outcomes [149]. With the increased number of disease models being investigated to assess the potential of allogeneic MSC treatment, and the increased sensitivity of techniques being utilized, the limits of immune modulation by allogeneic MSCs are emerging. These limits will most likely influence the disease targets suitable for cell therapy and help guide the next generation of enhanced allogeneic MSC therapies [177].

## References


1 Friedenstein, A.J., Piatetzky, S., II, and Petrakova, K.V. Osteogenesis in transplants of bone marrow cells. *J Embryol Exp Morphol* **16**,381–390 (1966).
2 Till, J.E. and McCulloch, E.A. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. *Radiat Res* **14**,213–222 (1961).
3 Friedenstein, A.J., Chailakhjan, R.K., and Lalykina, K.S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**,393–403 (1970).
4 Friedenstein, A.J., Chailakhyan, R.K., Latsinik, *et al.* Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. *Transplantation* **17**,331–340 (1974).
5 Friedenstein, A.J. Marrow stromal fibroblasts. *Calcif Tissue Int* **56** (Suppl 1),S17 (1995).
6 Owen, M. and Friedenstein, A.J. Stromal stem cells: marrow-derived osteogenic precursors. *Ciba Found Symp* **136**,42–60 (1988).
7 Caplan, A.I. Mesenchymal stem cells. *J Orthop Res* **9**,641–650 (1991).
8 Kuznetsov, S.A., Riminucci, M., Ziran, N., *et al.* The interplay of osteogenesis and hematopoiesis: expression of a constitutively active PTH/PTHrP receptor in osteogenic cells perturbs the establishment of hematopoiesis in bone and of skeletal stem cells in the bone marrow. *J Cell Biol* **167**,1113–1122 (2004).
9 Sacchetti, B., Funari, A., Michienzi, S., *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* **131**,324–336 (2007).
10 Chan, C.K., Seo, E.Y., Chen, J.Y., *et al.* Identification and specification of the mouse skeletal stem cell. *Cell* **160**,285–298 (2015).
11 Barry, F.P. and Murphy, J.M. Mesenchymal stem cells: clinical applications and biological characterization. *Int J Biochem Cell Biol* **36**,568–584 (2004).
12 Barry, F. and Murphy, M. Mesenchymal stem cells in joint disease and repair. *Nat Rev Rheumatol* **9**,584–594 (2013).
13 Bunnell, B.A., Estes, B.T., Guilak, F., and Gimble, J.M. Differentiation of adipose stem cells. *Methods Mol Biol* **456**,155–171 (2008).
14 Gimble, J.M., Katz, A.J., and Bunnell, B.A. Adipose-derived stem cells for regenerative medicine. *Circ Res* **100**,1249–1260 (2007).
15 Meliga, E., Strem, B.M., Duckers, H.J., and Serruys, P.W. Adipose-derived cells. *Cell Transplant* **16**,963–970 (2007).
16 Troyer, D.L. and Weiss, M.L. Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* **26**,591–599 (2008).
17 Weiss, M.L., Medicetty, S., Bledsoe, A.R., *et al.* Human umbilical cord matrix stem cells: preliminary characterization and effect of transplantation in a rodent model of Parkinson's disease. *Stem Cells* **24**,781–792 (2006).
18 Weiss, M.L. and Troyer, D.L. Stem cells in the umbilical cord. *Stem Cell Rev* **2**,155–162 (2006).
19 Flynn, A., Barry, F., and O'Brien, T. UC blood-derived mesenchymal stromal cells: an overview. *Cytotherapy* **9**,717–726 (2007).
20 Crisan, M., Yap, S., Casteilla, L., *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* **3**,301–313 (2008).
21 De Bari, C., Dell'Accio, F., Tylzanowski, P., and Luyten, F.P. Multipotent mesenchymal stem cells from adult human synovial membrane. *Arthritis Rheum* **44**,1928–1942 (2001).
22 Dowthwaite, G.P., Bishop, J.C., Redman, S.N., *et al.* The surface of articular cartilage contains a progenitor cell population. *J Cell Sci* **117**,889–897 (2004).
23 Khan, I.M., Bishop, J.C., Gilbert, S., and Archer, C.W. Clonal chondroprogenitors maintain telomerase activity and Sox9 expression during extended monolayer culture and retain chondrogenic potential. *Osteoarthritis Cartilage* **17**,518–528 (2009).
24 Huang, T.F., Chen, Y.T., Yang, T.H., *et al.* Isolation and characterization of mesenchymal stromal cells from human anterior cruciate ligament. *Cytotherapy* **10**,806–814 (2008).
25 Steinert, A.F., Kunz, M., Prager, P., *et al.* Mesenchymal stem cell characteristics of human anterior cruciate ligament outgrowth cells. *Tissue Eng Part A* **17**,1375–1388 (2011).
26 Murray, M.M., Bennett, R., Zhang, X., and Spector, M. Cell outgrowth from the human ACL in vitro: regional variation and response to TGF-β1. *J Orthop Res* **20**,875–880 (2002).
27 Murray, M.M. and Spector, M. The migration of cells from the ruptured human anterior cruciate ligament into collagen-glycosaminoglycan regeneration templates *in vitro*. *Biomaterials* **22**,2393–2402 (2001).
28 Cheng, M.T., Yang, H.W., Chen, T.H., and Lee, O.K. Isolation and characterization of multipotent stem cells from human cruciate ligaments. *Cell Prolif* **42**,448–460 (2009).
29 Sakaguchi, Y., Sekiya, I., Yagishita, K., and Muneta, T. Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source. *Arthritis Rheum* **52**,2521–2529 (2005).
30 Fan, J., Varshney, R.R., Ren, L., *et al.* Synovium-derived mesenchymal stem cells: a new cell source for musculoskeletal regeneration. *Tissue Eng Part B Rev* **15**,75–86 (2009).
31 Tintut, Y., Alfonso, Z., Saini, T., *et al.* Multilineage potential of cells from the artery wall. *Circulation* **108**,2505–2510 (2003).
32 Mody, N., Tintut, Y., Radcliff, K., and Demer, L.L. Vascular calcification and its relation to bone calcification: possible underlying mechanisms. *J Nucl Cardiol* **10**,177–183 (2003).
33 Tavian, M., Zheng, B., Oberlin, E., *et al.* The vascular wall as a source of stem cells. *Ann N Y Acad Sci* **1044**,41–50 (2005).
34 Abedin, M., Tintut, Y., and Demer, L.L. Mesenchymal stem cells and the artery wall. *Circ Res* **95**,671–676 (2004).
35 Da Silva Meirelles, L., Chagastelles, P.C., and Nardi, N.B. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* **119**,2204–2213 (2006).
36 Shi, S. and Gronthos, S. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* **18**,696–704 (2003).
37 Tavian, M. and Peault, B. The changing cellular environments of hematopoiesis in human development in utero. *Exp Hematol* **33**,1062–1069 (2005).
38 Zannettino, A.C., Patton, S., Arthur, A., *et al.* Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype *in vitro* and *in vivo*. *J Cell Physiol* **214**,413–421 (2008).
39 Caplan, A.I. All MSCs are pericytes? *Cell Stem Cell* **3**,229–230 (2008).
40 Kurth, T.B., Dell'accio, F., Crouch, V., *et al.* Functional mesenchymal stem cell niches in adult mouse knee joint synovium *in vivo*. *Arthritis Rheum* **63**,1289–1300 (2011).
41 Reinisch, A., Etchart, N., Thomas, D., *et al.* Epigenetic and *in vivo* comparison of diverse MSC sources reveals an endochondral

signature for human hematopoietic niche formation. *Blood* **125**,249–260 (2015).
42 Rinkevich, Y., Lindau, P., Ueno, H., *et al.* Germ-layer and lineage-restricted stem/progenitors regenerate the mouse digit tip. *Nature* **476**,409–413 (2011).
43 Park, D., Spencer, J.A., Koh, B.I., *et al.* Endogenous bone marrow MSCs are dynamic, fate-restricted participants in bone maintenance and regeneration. *Cell Stem Cell* **10**,259–272 (2012).
44 Isern, J., García-García, A., Martín, A.M., *et al.* The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *Elife* **3**,e03696 (2014).
45 Mendez-Ferrer, S., Scadden, D.T., and Sanchez-Aguilera, A. Bone marrow stem cells: current and emerging concepts. *Ann N Y Acad Sci* **1335**,32–44 (2015).
46 Mendez-Ferrer, S., Michurina, T.V., Ferraro, F., *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* **466**,829–834 (2010).
47 Takashima, Y., Era, T., Nakao, K., *et al.* Neuroepithelial cells supply an initial transient wave of MSC differentiation. *Cell* **129**,1377–1388 (2007).
48 Pevsner-Fischer, M., Levin, S., and Zipori, D. The origins of mesenchymal stromal cell heterogeneity. *Stem Cell Rev* **7**,560–568 (2011).
49 Phinney, D.G. Functional heterogeneity of mesenchymal stem cells: implications for cell therapy. *J Cell Biochem* **113**,2806–2812 (2012).
50 Pacini, S. Deterministic and stochastic approaches in the clinical application of mesenchymal stromal cells (MSCs). *Front Cell Dev Biol* **2**,50 (2014).
51 Russell, K.C., Phinney, D.G., Lacey, M.R., *et al. In vitro* high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells* **28**,788–798 (2010).
52 Harrington, J., Sloan, A.J., and Waddington, R.J. Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow. *Connect Tissue Res* **55**(Suppl 1),62–67 (2014).
53 Kuci, Z., Seiberth, J., Latifi-Pupovci, H., *et al.* Clonal analysis of multipotent stromal cells derived from $CD271^+$ bone marrow mononuclear cells: functional heterogeneity and different mechanisms of allosuppression. *Haematologica* **98**,1609–1616 (2013).
54 Muraglia, A., Cancedda, R., and Quarto, R. Clonal mesenchymal progenitors from human bone marrow differentiate in vitro according to a hierarchical model. *J Cell Sci* **113**(Pt 7)1161–1166 (2000).
55 Pittenger, M.F., Mackay, A.M., Beck, S.C., *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* **284**,143–147 (1999).
56 Delorme, B. and Charbord, P. Culture and characterization of human bone marrow mesenchymal stem cells. *Methods Mol Med* **140**,67–81 (2007).
57 Bianco, P., Robey, P.G., and Simmons, P.J. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* **2**,313–319 (2008).
58 Murphy, M. and Barry, F. Cellular chondroplasty: a new technology for joint regeneration. *J Knee Surg* **28**,45–50 (2015).
59 Ankrum, J. and Karp, J.M. Mesenchymal stem cell therapy: two steps forward, one step back. *Trends Mol Med* **16**,203–209 (2010).
60 Farini, A., Sitzia, C., Erratico, S., *et al.* Clinical applications of mesenchymal stem cells in chronic diseases. *Stem Cells Int* **2014**,306573 (2014).
61 Sharma, R.R., Pollock, K., Hubel, A., and McKenna, D. Mesenchymal stem or stromal cells: a review of clinical applications and manufacturing practices. *Transfusion* **54**,1418–1437 (2014).
62 Sutton, M.T. and Bonfield, T.L. Stem cells: innovations in clinical applications. *Stem Cells Int* **2014**,516278 (2014).
63 Hoch, A.I. and Leach, J.K. Concise review: optimizing expansion of bone marrow mesenchymal stem/stromal cells for clinical applications. *Stem Cells Transl Med* **3**,643–652 (2014).
64 Friedenstein, A.J., Petrakova, K.V., Kurolesova, A.I., and Frolova, G.P. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* **6**,230–247 (1968).
65 Quarto, R., Mastrogiacomo, M., Cancedda, R., *et al.* Repair of large bone defects with the use of autologous bone marrow stromal cells. *N Engl J Med* **344**,385–386 (2001).
66 Bruder, S.P., Jaiswal, N., Ricalton, N.S., *et al.* Mesenchymal stem cells in osteobiology and applied bone regeneration. *Clin Orthop Relat Res* (355 Suppl),S247–S256 (1998).
67 Bruder, S.P., Kraus, K.H., Goldberg, V.M., and Kadiyala, S. The effect of implants loaded with autologous mesenchymal stem cells on the healing of canine segmental bone defects. *J Bone Joint Surg Am* **80**,985–996 (1998).
68 Bruder, S.P., Kurth, A.A., Shea, M., *et al.* Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *J Orthop Res* **16**,155–162 (1998).
69 Bruder, S.P., Fink, D.J., and Caplan, A.I. Mesenchymal stem cells in bone development, bone repair, and skeletal regeneration therapy. *J Cell Biochem* **56**,283–294 (1994).
70 Young, R.G., Butler, D.L., Weber, W., *et al.* Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res* **16**,406–413 (1998).
71 Awad, H.A., Butler, D.L., Boivin, G.P., *et al.* Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng* **5**,267–277 (1999).
72 Wakitani, S., Goto, T., Pineda, S.J., *et al.* Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* **76**,579–592 (1994).
73 Grande, D.A., Southerland, S.S., Manji, R., *et al.* Repair of articular cartilage defects using mesenchymal stem cells. *Tissue Eng* **1**,345–353 (1995).
74 Cinotti, G., Patti, A.M., Vulcano, A., *et al.* Experimental posterolateral spinal fusion with porous ceramics and mesenchymal stem cells. *J Bone Joint Surg Br* **86**,135–142 (2004).
75 Dumont, R.J., Dayoub, H., Li, J.Z., *et al. Ex vivo* bone morphogenetic protein-9 gene therapy using human mesenchymal stem cells induces spinal fusion in rodents. *Neurosurgery* **51**,1239–1244, discussion 1244–1235 (2002).
76 Minamide, A., Yoshida, M., Kawakami, M., *et al.* The use of cultured bone marrow cells in type I collagen gel and porous hydroxyapatite for posterolateral lumbar spine fusion. *Spine (Phila Pa 1976)* **30**,1134–1138 (2005).
77 Horwitz, E.M., Gordon, P.L., Koo, W.K., *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci U S A* **99**,8932–8937 (2002).
78 Horwitz, E.M., Prockop, D.J., Fitzpatrick, L.A., *et al.* Transplantability and therapeutic effects of bone marrow-derived

mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* **5**,309–313 (1999).
79 Horwitz, E.M., Prockop, D.J., Gordon, P.L., *et al.* Clinical responses to bone marrow transplantation in children with severe osteogenesis imperfecta. *Blood* **97**,1227–1231 (2001).
80 Le Blanc, K., Götherström, C., Ringdén, O., *et al.* Fetal mesenchymal stem-cell engraftment in bone after *in utero* transplantation in a patient with severe osteogenesis imperfecta. *Transplantation* **79**,1607–1614 (2005).
81 Murphy, J.M., Fink, D.J., Hunziker, E.B., and Barry, F.P. Stem cell therapy in a caprine model of osteoarthritis. *Arthritis Rheum* **48**,3464–3474 (2003).
82 Toma, C., Pittenger, M.F., Cahill, K.S., *et al.* Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**,93–98 (2002).
83 Caplan, A.I. and Dennis, J.E. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* **98**1076–1084 (2006).
84 Lee, R.H., Pulin, A.A., Seo, M.J., *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* **5**,54–63 (2009).
85 Higman, V.A., Briggs, D.C., Mahoney, D.J., *et al.* A refined model for the TSG-6 link module in complex with hyaluronan: use of defined oligosaccharides to probe structure and function. *J Biol Chem* **289**,5619–5634 (2014).
86 Dyer, D.P., Thomson, J.M., Hermant, A., *et al.* TSG-6 inhibits neutrophil migration via direct interaction with the chemokine CXCL8. *J Immunol* **192**,2177–2185 (2014).
87 Choi, H., Lee, R.H., Bazhanov, N., *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood* **118**,330–338 (2011).
88 Gnecchi, M., He, H., Liang, O.D., *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* **11**,367–368 (2005).
89 Gnecchi, M., He, H., Noiseux, N., *et al.* Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* **20**,661–669 (2006).
90 Mirotsou, M., Zhang, Z., Deb, A., *et al.* Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* **104**,1643–1648 (2007).
91 Hankenson, K.D. and Bornstein, P. The secreted protein thrombospondin 2 is an autocrine inhibitor of marrow stromal cell proliferation. *J Bone Miner Res* **17**,415–425 (2002).
92 Jeong, S.Y., Kim, D.H., Ha, J., *et al.* Thrombospondin-2 secreted by human umbilical cord blood-derived mesenchymal stem cells promotes chondrogenic differentiation. *Stem Cells* **31**,2136–2148 (2013).
93 Maumus, M., Manferdini, C., Toupet, K., *et al.* Adipose mesenchymal stem cells protect chondrocytes from degeneration associated with osteoarthritis. *Stem Cell Res* **11**,834–844 (2013).
94 Schelbergen, R.F., van Dalen, S., ter Huurne, M., *et al.* Treatment efficacy of adipose-derived stem cells in experimental osteoarthritis is driven by high synovial activation and reflected by S100A8/A9 serum levels. *Osteoarthritis Cartilage* **22**,1158–1166 (2014).
95 Friedenstein, A.J., Gorskaja, J.F., and Kulagina, N.N. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* **4**,267–274 (1976).
96 Mahmood, A., Lu, D., Lu, M., and Chopp, M. Treatment of traumatic brain injury in adult rats with intravenous administration of human bone marrow stromal cells. *Neurosurgery* **53**,697–702, discussion 702–693 (2003).
97 Bartholomew, A., Sturgeon, C., Siatskas, M., *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* **30**,42–48 (2002).
98 Di Nicola, M., Carlo-Stella, C., Magni, M., *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* **99**,3838–3843 (2002).
99 English, K. Mechanisms of mesenchymal stromal cell immunomodulation. *Immunol Cell Biol* **91**,19–26 (2013).
100 Uccelli, A., Moretta, L., and Pistoia, V. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* **8**,726–736 (2008).
101 Wang, Y., Chen, X., Cao, W., and Shi, Y. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* **15**,1009–1016 (2014).
102 Bernardo, M.E. and Fibbe, W.E. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* **13**,392–402 (2013).
103 Le Blanc, K. and Mougiakakos, D. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* **12**,383–396 (2012).
104 Bernardo, M.E., Ball, L.M., Cometa, A.M., *et al.* Co-infusion of *ex vivo*-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. *Bone Marrow Transplant* **46**,200–207 (2011).
105 Le Blanc, K., Rasmusson, I., Sundberg, B., *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* **363**,1439–1441 (2004).
106 Tobin, L.M., Healy, M.E., English, K., and Mahon, B.P. Human mesenchymal stem cells suppress donor $CD4^+$ T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease. *Clin Exp Immunol* **172**,333–348 (2013).
107 Huang, X.P., Sun, Z., Miyagi, Y., *et al.* Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation* **122**,2419–2429 (2010).
108 Al Jumah, M.A. and Abumaree, M.H. The immunomodulatory and neuroprotective effects of mesenchymal stem cells (MSCs) in experimental autoimmune encephalomyelitis (EAE): a model of multiple sclerosis (MS). *Int J Mol Sci* **13**,9298–9331 (2012).
109 Bowles, A.C., Scruggs, B.A., and Bunnell, B.A. Mesenchymal stem cell-based therapy in a mouse model of experimental autoimmune encephalomyelitis (EAE). *Methods Mol Biol* **1213**,303–319 (2014).
110 Matysiak, M., Orlowski, W., Fortak-Michalska, M., *et al.* Immunoregulatory function of bone marrow mesenchymal stem cells in EAE depends on their differentiation state and secretion of PGE2. *J Neuroimmunol* **233**,106–111 (2011).
111 Perico, N., Casiraghi, F., Introna, M., *et al.* Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* **6**,412–422 (2011).

112 Treacy, O., O'Flynn, L., Ryan, A.E., *et al.* Mesenchymal stem cell therapy promotes corneal allograft survival in rats by local and systemic immunomodulation. *Am J Transplant* **14**,2023–2036 (2014).

113 Liang, J., Li, X., Zhang, H., *et al.* Allogeneic mesenchymal stem cells transplantation in patients with refractory RA. *Clin Rheumatol* **31**,157–161 (2012).

114 Harada, N., Watanabe, Y., Sato, K., *et al.* Bone regeneration in a massive rat femur defect through endochondral ossification achieved with chondrogenically differentiated MSCs in a degradable scaffold. *Biomaterials* **35**,7800–7810 (2014).

115 Voswinkel, J., Francois, S., Simon, J.M., *et al.* Use of mesenchymal stem cells (MSC) in chronic inflammatory fistulizing and fibrotic diseases: a comprehensive review. *Clin Rev Allergy Immunol* **45**,180–192 (2013).

116 Duijvestein, M., Wildenberg, M.E., Welling, M.M., *et al.* Pretreatment with interferon-γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells* **29**,1549–1558 (2011).

117 Duijvestein, M., Vos, A.C., Roelofs, H., *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* **59**,1662–1669 (2010).

118 Curley, G.F., Ansari, B., Hayes, M., *et al.* Effects of intratracheal mesenchymal stromal cell therapy during recovery and resolution after ventilator-induced lung injury. *Anesthesiology* **118**,924–932 (2013).

119 Lalu, M.M., McIntyre, L., Pugliese, C., *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* **7**,e47559 (2012).

120 Kolf, C.M., Cho, E., and Tuan, R.S. Mesenchymal stromal cells. Biology of adult mesenchymal stem cells: regulation of niche, self-renewal and differentiation. *Arthritis Res Ther* **9**,204 (2007).

121 Glenn, J.D. and Whartenby, K.A. Mesenchymal stem cells: emerging mechanisms of immunomodulation and therapy. *World J Stem Cells* **6**,526–539 (2014).

122 Singer, N.G. and Caplan, A.I. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol* **6**,457–478 (2011).

123 Ren, G., Zhang, L., Zhao, X., *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* **2**,141–150 (2008).

124 Lee, S., Szilagyi, E., Chen, L., *et al.* Activated mesenchymal stem cells increase wound tensile strength in aged mouse model via macrophages. *J Surg Res* **181**,20–24 (2013).

125 Waterman, R.S., Tomchuck, S.L., Henkle, S.L., and Betancourt, A.M. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* **5**,e10088 (2010).

126 English, K., Barry, F.P., Field-Corbett, C.P., and Mahon, B.P. IFN-γ and TNF-α differentially regulate immunomodulation by murine mesenchymal stem cells. *Immunol Lett* **110**,91–100 (2007).

127 Krampera, M. Mesenchymal stromal cell "licensing": a multistep process. *Leukemia* **25**,1408–1414 (2011).

128 Sivanathan, K.N., Gronthos, S., Rojas-Canales, D., *et al.* Interferon-γ modification of mesenchymal stem cells: implications of autologous and allogeneic mesenchymal stem cell therapy in allotransplantation. *Stem Cell Rev* **10**,351–375 (2014).

129 Griffin, M.D., Ritter, T., and Mahon, B.P. Immunological aspects of allogeneic mesenchymal stem cell therapies. *Hum Gene Ther* **21**,1641–1655 (2010).

130 Gupta, N., Su, X., Popov, B., *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* **179**,1855–1863 (2007).

131 Raffaghello, L., Bianchi, G., Bertolotto, M., *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* **26**,151–162 (2008).

132 Su, W.R., Zhang, Q.Z., Shi, S.H., *et al.* Human gingiva-derived mesenchymal stromal cells attenuate contact hypersensitivity via prostaglandin E2-dependent mechanisms. *Stem Cells* **29**,1849–1860 (2011).

133 Bouffi, C., Bony, C., Courties, G., *et al.* IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. *PLoS ONE* **5**,e14247 (2010).

134 English, K., Barry, F.P., and Mahon, B.P. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Lett* **115**,50–58 (2008).

135 Djouad, F., Charbonnier, L.M., Bouffi, C., *et al.* Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* **25**,2025–2032 (2007).

136 Nauta, A.J., Kruisselbrink, A.B., Lurvink, E., *et al.* Mesenchymal stem cells inhibit generation and function of both $CD34^{+}$-derived and monocyte-derived dendritic cells. *J Immunol* **177**,2080–2087 (2006).

137 Chiesa, S., Morbelli, S., Morando, S., *et al.* Mesenchymal stem cells impair in vivo T-cell priming by dendritic cells. *Proc Natl Acad Sci U S A* **108**,17384–17389 (2011).

138 Zhang, B., Liu, R., Shi, D., *et al.* Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* **113**,46–57 (2009).

139 Ge, W., Jiang, J., Baroja, M.L., *et al.* Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *Am J Transplant* **9**,1760–1772 (2009).

140 Liu, X., Qu, X., Chen, Y., *et al.* Mesenchymal stem/stromal cells induce the generation of novel IL-10-dependent regulatory dendritic cells by SOCS3 activation. *J Immunol* **189**,1182–1192 (2012).

141 Duffy, M.M., Ritter, T., Ceredig, R., and Griffin, M.D. Mesenchymal stem cell effects on T-cell effector pathways. *Stem Cell Res Ther* **2**,34 (2011).

142 Cutler, A.J., Limbani, V., Girdlestone, J., and Navarrete, C.V. Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. *J Immunol* **185**,6617–6623 (2010).

143 Selmani, Z., Naji, A., Zidi, I., *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^{+}CD25^{high}FOXP3^{+}$ regulatory T cells. *Stem Cells* **26**,212–222 (2008).

144 Melief, S.M., Schrama, E., Brugman, M.H., *et al.* Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells* **31**,1980–1991 (2013).

145 Melief, S.M., Geutskens, S.B., Fibbe, W.E., and Roelofs, H. Multipotent stromal cells skew monocytes towards an anti-inflammatory interleukin-10-producing phenotype by production of interleukin-6. *Haematologica* **98**,888–895 (2013).

146 Kavanagh, H. and Mahon, B.P. Allogeneic mesenchymal stem cells prevent allergic airway inflammation by inducing murine regulatory T cells. *Allergy* **66**,523–531 (2011).

147 Nemeth, K., Keane-Myers, A., Brown, J.M., *et al.* Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci U S A* **107**,5652–5657 (2010).

148 Akiyama, K., Chen, C., Wang, D., *et al.* Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* **10**,544–555 (2012).

149 Griffin, M.D., Ryan, A.E., Alagesan, S., *et al.* Anti-donor immune responses elicited by allogeneic mesenchymal stem cells: what have we learned so far? *Immunol Cell Biol* **91**,40–51 (2013).

150 Barry, F.P., Murphy, J.M., English, K., and Mahon, B.P. Immunogenicity of adult mesenchymal stem cells: lessons from the fetal allograft. *Stem Cells Dev* **14**,252–265 (2005).

151 Zheng, Z.H., Li, X.Y., Ding, J., *et al.* Allogeneic mesenchymal stem cell and mesenchymal stem cell-differentiated chondrocyte suppress the responses of type II collagen-reactive T cells in rheumatoid arthritis. *Rheumatology (Oxford)* **47**,22–30 (2008).

152 Weyand, C.M. and Goronzy, J.J. Stem cell aging and autoimmunity in rheumatoid arthritis. *Trends Mol Med* **10**,426–433 (2004).

153 Murphy, J.M., Dixon, K., Beck, S., *et al.* Reduced chondrogenic and adipogenic activity of mesenchymal stem cells from patients with advanced osteoarthritis. *Arthritis Rheum* **46**,704–713 (2002).

154 Camp, D.M., Loeffler, D.A., Farrah, D.M., *et al.* Cellular immune response to intrastriatally implanted allogeneic bone marrow stromal cells in a rat model of Parkinson's disease. *J Neuroinflammation* **6**,17 (2009).

155 Chen, L., Tredget, E.E., Liu, C., and Wu, Y. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. *PLoS ONE* **4**,e7119 (2009).

156 Zangi, L., Margalit, R., Reich-Zeliger, S., *et al.* Direct imaging of immune rejection and memory induction by allogeneic mesenchymal stromal cells. *Stem Cells* **27**,2865–2874 (2009).

157 Tögel, F., Cohen, A., Zhang, P., *et al.* Autologous and allogeneic marrow stromal cells are safe and effective for the treatment of acute kidney injury. *Stem Cells Dev* **18**,475–485 (2009).

158 Nauta, A.J., Westerhuis, G., Kruisselbrink, A.B., *et al.* Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* **108**,2114–2120 (2006).

159 Rafei, M., Birman, E., Forner, K., and Galipeau, J. Allogeneic mesenchymal stem cells for treatment of experimental autoimmune encephalomyelitis. *Mol Ther* **17**,1799–1803 (2009).

160 Seifert, M., Stolk, M., Polenz, D., and Volk, H.D. Detrimental effects of rat mesenchymal stromal cell pre-treatment in a model of acute kidney rejection. *Front Immunol* **3**,202 (2012).

161 Isakova, I.A., Dufour, J., Lanclos, C., *et al.* Cell-dose-dependent increases in circulating levels of immune effector cells in rhesus macaques following intracranial injection of allogeneic MSCs. *Exp Hematol* **38**,957–967.e1 (2010).

162 Poncelet, A.J., Vercruysse, J., Saliez, A., and Gianello, P. Although pig allogeneic mesenchymal stem cells are not immunogenic *in vitro,* intracardiac injection elicits an immune response *in vivo*. *Transplantation* **83**,783–790 (2007).

163 Badillo, A.T., Beggs, K.J., Javazon, E.H., *et al.* Murine bone marrow stromal progenitor cells elicit an *in vivo* cellular and humoral alloimmune response. *Biol Blood Marrow Transplant* **13**,412–422 (2007).

164 Beggs, K.J., Lyubimov, A., Borneman, J.N., *et al.* Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. *Cell Transplant* **15**,711–721 (2006).

165 Eliopoulos, N., Stagg, J., Lejeune, L., *et al.* Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* **106**,4057–4065 (2005).

166 Griffin, M.D., Elliman, S.J., Cahill, E., *et al.* Concise review: adult mesenchymal stromal cell therapy for inflammatory diseases: how well are we joining the dots? *Stem Cells* **31**,2033–2041 (2013).

167 Bernardo, M.E., Pagliara, D., and Locatelli, F. Mesenchymal stromal cell therapy: a revolution in regenerative medicine? *Bone Marrow Transplant* **47**,164–171 (2012).

168 Wang, S., Qu, X., and Zhao, R.C. Clinical applications of mesenchymal stem cells. *J Hematol Oncol* **5**,19 (2012).

169 Stegall, M.D., Dean, P.G., and Gloor, J. Mechanisms of alloantibody production in sensitized renal allograft recipients. *Am J Transplant* **9**,998–1005 (2009).

170 Krampera, M., Cosmi, L., Angeli, R., *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* **24**,386–398 (2006).

171 Schu, S., Nosov, M., O'Flynn, L., *et al.* Immunogenicity of allogeneic mesenchymal stem cells. *J Cell Mol Med* **16**,2094–2103 (2012).

172 Polchert, D., Sobinsky, J., Douglas, G., *et al.* IFN-γ activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. *Eur J Immunol* **38**,1745–1755 (2008).

173 Ankrum, J.A., Ong, J.F., and Karp, J.M. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotechnol* **32**,252–260 (2014).

174 Lohan, P., Coleman, C.M., Murphy, J.M., *et al.* Changes in immunological profile of allogeneic mesenchymal stem cells after differentiation: should we be concerned? *Stem Cell Res Ther* **5**,99 (2014).

175 Ryan, A.E., Lohan, P., O'Flynn, L., *et al.* Chondrogenic differentiation increases antidonor immune response to allogeneic mesenchymal stem cell transplantation. *Mol Ther* **22**,655–667 (2014).

176 Dhingra, S., Li, P., Huang, X.P., *et al.* Preserving prostaglandin E2 level prevents rejection of implanted allogeneic mesenchymal stem cells and restores postinfarction ventricular function. *Circulation* **128**,S69–S78 (2013).

177 English, K. and Mahon, B.P. Allogeneic mesenchymal stem cells: agents of immune modulation. *J Cell Biochem* **112**,1963–1968 (2011).

CHAPTER 30

# The differences between mesenchymal stromal cells and fibroblasts

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## 30.1 Introduction

Cellular therapy is a promising tool to treat a wide plethora of human diseases. So far, extensive studies have been done, and are still continuing to be done, to better characterize many aspects of cellular therapy for regenerative medicine purposes. Cell types proposed as potential candidates for cellular therapy can be classified into three groups: embryonic stem cells (ESCs) [1], induced pluripotent stem cells (iPSCs) [2], and adult mesenchymal stromal cells (MSCs) [3]. Although derived in different ways, both ECSs and iPSCs are capable of extensive self-renewal and expansion, and have the potential to differentiate into any type of somatic cell [4,5]. However, there are limitations to be considered for their clinical use, ranging from the ethical issues surrounding ESCs – primarily the destruction of the embryo by harvesting the cells early in embryonic development – difficulties in genetically manipulating iPSCs, immune reactions to the differentiated progeny of ESCs and iPSCs if derived from an MHC-incompatible donor, and, importantly, teratoma formation after transplantation of the differentiated progeny of ESCs or iPSCs if they are contaminated with undifferentiated ESCs or iPSCs [6,7].

MSCs were first isolated by Friedenstein from bone marrow (BM) [8]. Later, these cells were also isolated from multiple different human adult tissues, including umbilical cord blood (UCB) [9], skeletal muscle [10], skin [11], periodontal ligament [12], and adipose tissue (AT) [13]. MSCs are considered an optimal candidate cell population for cellular therapy as they have been reported to possess multipotent differentiation potential of mesodermal lineages [14], paracrine functions [15], genetic stability after *in vitro* manipulation [16], and, most importantly, MSCs have no ethical or manufacturing issues compared with ESCs and iPSCs.

One unsolved issue to ensure an efficient and reproducible use of MSCs in clinical practice is evaluation of their purity. The absence of specific markers that characterize MSCs is responsible for this. The presence of different cell types other than MSCs in a given preparation of cells such as hematopoietic and endothelial cells can be easily evaluated by the expression of CD34, CD45, and CD31 markers that are not present in MSCs [17]. In contrast, the occurrence of contaminating fibroblasts is hard to establish; thus, the assessment of the homogeneity and purity of an MSC preparation remains difficult. In contrast to MSCs, fibroblasts are considered mature MSCs that are particularly abundant in the connective tissue of each organ. Several findings have indicated

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that MSCs and fibroblasts share many phenotypic and functional characteristics besides their morphology and origin [18–20]. For this reason, ready discrimination between MSCs and fibroblasts is difficult. It has been demonstrated that the presence of contaminating fibroblasts can represent a serious obstacle in determining the therapeutic potential of MSCs. It has been recently shown that co-administration of MSCs with contaminating fibroblasts in an experimental model of Parkinson's disease could have detrimental effects [21]. It is thus important to further distinguish the phenotypic and functional characteristics of MSCs and their differences compared with fibroblasts.

Although both cell populations have similarities, in this chapter we will describe some aspects and properties that can lead to a better definition of the differences between MSCs and fibroblasts.

## 30.2 Phenotypic similarities and differences between mesenchymal stromal cells and fibroblasts

Three important features have been suggested as characteristics of human MSCs: adherence to plastic in tissue culture, a characteristic cell surface antigen expression, and multipotent differentiation potential along the mesodermal lineages [22]. It is immediately apparent that the first criterion is not useful for distinguishing MSCs from fibroblasts. Indeed, as shown in Figure 30.1, both fibroblasts and MSCs adhere to plastic and have a spindle-shaped morphology.

Additionally, mesodermal lineage transdifferentiation potential is not an exclusive property of MSCs, since many studies have demonstrated that both MSCs and fibroblasts show a similar capacity to differentiate mesodermally.

## 30.3 Cell surface membrane markers

A minimum set of phenotypic criteria have been proposed to define MSCs: the presence (≥95%) of the three surface markers CD105, CD73, and CD90 and the absence (≤2% positive) of CD45, CD34, CD31, CD14 or CD11b, CD79a or CD19, and human leukocyte antigen (HLA) class II [22]. Other surface markers, such as CD13, CD29, CD44, and CD10, are variably expressed on MSCs [23]. However, these minimal criteria are insufficient to ascertain the purity of an MSC population because it has been shown that fibroblasts, at various stages, express all these surface markers [19,24]. For this reason, many investigators have tried to identify other markers that could be used to better define an MSC population [25]. Table 30.1 lists some cell surface membrane molecules that appear to be expressed on MSCs but not on fibroblasts.

Stro-1 is one of the best known markers for MSCs but it is not universally expressed on all MSC populations. Indeed, AT-derived MSCs, UCB-derived MSCs, and peripheral blood-derived MSCs have no or low Stro-1 expression [26–28]. Controversial data exist about Stro-1 expression by fibroblasts. Chen *et al* [29]. reported that Stro-1 was expressed by fibroblasts, albeit at low levels. Conversely, other authors demonstrated that fibroblasts do not express Stro-1 [19,30].

CD271 is an MSC marker used to select fibroblastic colony-forming units (CFU-Fs) from BM mononuclear cells. Similar to Stro-1, CD271 is not expressed by all MSCs. Indeed, its expression was found elevated in BM-MSCs and AT-MSCs [31,32], but at low levels in placenta-derived MSCs and totally absent in peripheral blood-derived MSCs [33]. Interestingly, Wirths *et al.* found that CD271 was expressed on BM-MSCs but not on foreskin fibroblasts [34].

The CD146 cell adhesion protein plays a pivotal role in vascular endothelial cell activity and angiogenesis. CD146 is

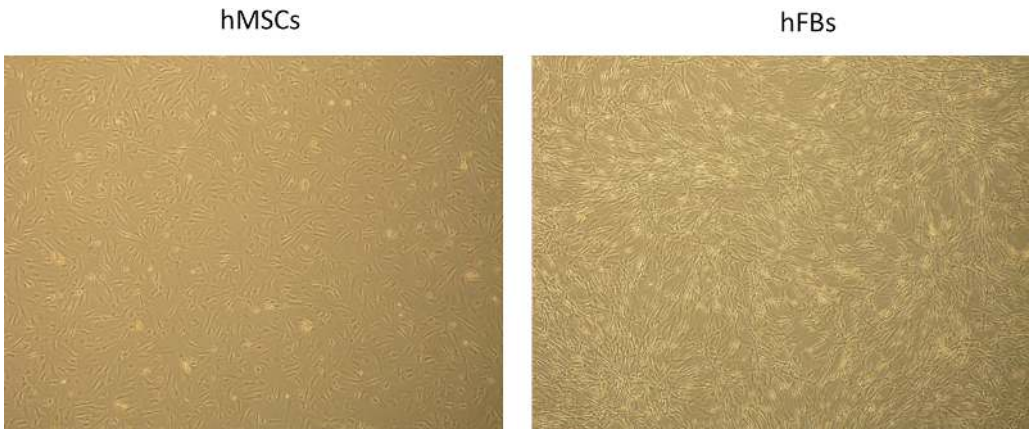



**Figure 30.1** Cell morphology of human MSCs (hMSCs) and human fibroblasts (hFBs). Representative photographs of MSCs isolated from AT and an available commercial line of foreskin fibroblasts. MSCs share a splindle-shaped fibroblasts-like morphology. Cell culture photographs were acquired at passage 5 for both cell populations.

**Table 30.1** Surface molecules identified on MSC cell populations and absent on fibroblasts.

| Marker | Main function or description | MSCs | Fibroblasts |
|---|---|---|---|
| Stro-1 | Cell membrane stromal cell antigen 1, which translocates from the endoplasmic reticulum to the cell membrane in response to a depletion of intracellular calcium | Yes [26–28] | No [19,29,30][a] |
| CD271 | Receptor for neurotrophin (low-affinity nerve growth factor receptor), which stimulates neuronal cells to survive and differentiate | Yes [31–33] | No [34] |
| CD146 | Plays a role in cell adhesion, and in cohesion of the endothelial monolayer at intercellular junctions in vascular tissue. Also known as melanoma cell adhesion molecule or cell surface glycoprotein MUC18 | Yes [35–40] | No [24] |
| CD49f | $\alpha_6$-Integrin, involved in cell adhesion as well as cell-surface-mediated signaling | Yes [41–43] | No [44] |
| GD2 | Neural ganglioside | Yes [45,46] | No [45] |
| MSCA-1 (TNAP, W8B2) | MSCA-1 is identical to TNAP | Yes [23,35,47] | No [34] |
| CD200 | Type-1 membrane glycoprotein that may regulate myeloid cell activity and delivers an inhibitory signal for the macrophage lineage in diverse tissues | Yes [48] | No [49] |
| TM4SF1 | Protein belonging to the tetraspanin family. Mediates signal transduction events that play a role in the regulation of cell development, activation, growth, and motility | Yes [50] | No [50] |

MSCA-1: mesenchymal stem cell antigen-1; Stro-1: stromal precursor antigen-1; TM4SF1: transmembrane 4 L6 super family member; TNAP: tissue nonspecific alkaline phosphatase.
[a] According to [29], Stro-1 is expressed in fibroblasts.

expressed by several MSC populations, including BM-MSCs [35], AT-MSCs [36], UCB-MSCs [37], and MSCs isolated from placenta [38], dermis [39], and periodontal ligament [40], but not by fibroblasts [24].

CD49f ($\alpha_6$-integrin) expression has been detected in BM-MSCs [41], fetal urinary-bladder-derived MSCs [42], and UC-MSCs [43]. It seems not to be expressed by fibroblasts [44].

The neural ganglioside GD2 was reported by Martinez *et al.* to be expressed on BM-MSCs and AT-MSCs but not on foreskin fibroblasts [45]. Moreover, GD2 identified a subpopulation of UC-MSCs [46].

MSCA-1 (also named TNAP [47] or W8B8 [35]) has been reported as a suitable marker for the prospective isolation of highly purified BM-MSCs [23]. MSCA-1 expression is probably limited to BM-MSC subpopulations. Fibroblasts do not express this surface antigen [34].

CD200 is expressed on the surface of human MSCs. However, it also is not detected on fibroblasts [48,49].

TM4SF1 was recently described as being highly expressed by BM-, UC-, and AT-MSCs but not on fibroblasts [50].

Thus, in summary, some of the surface antigens described here, specifically CD271, CD146, GD2, MSCA-1, and CD200, appear to represent, at least in part, cell surface markers expressed by MSC or MSC subpopulations, but not by fibroblasts.

## 30.4 Gene expression profile of mesenchymal stromal cells and fibroblasts

The development of techniques such as microarray technology, quantitative real-time reverse transcriptase polymerase chain

reaction (qRT-PCR) and proteomics has allowed an in-depth analysis of cellular gene and protein profiling. Several studies have examined differential gene expression between MSCs and fibroblasts. Using DNA microarrays probing for 9400 genes, Ishii *et al* [51]. found that approximately 100 genes were differentially expressed between MSCs and fibroblasts and that these differences were not related to age, donor gender or cell culture conditions. Reverse transcriptase polymerase chain reaction (RT-PCR) and qRT-PCR showed that mRNA levels were expressed at a lower level in MSCs compared with fibroblasts for the following: matrix metalloproteinase (MMP)-1, adrenomedullin, CUG triplet repeat RNA-binding protein, collagen type XV α-1, KH domain-containing RNA-binding signal transduction-associated protein 3 (KHDRBS3), C-type lectin superfamily member-2, and apolipoprotein D.

The genes related to these mRNAs encode proteins with the following functions:

- Adrenomedullin is a ubiquitously expressed peptide hormone initially isolated from a pheochromyctoma, a tumor of the adrenal medulla.
- CUG triplet repeat RNA-binding protein is involved in the regulation of pre-mRNA alternative splicing and possibly in mRNA editing and translation.
- Collagen type XV α-1 is a member of the FACIT collagen family (fibril-associated collagens with interrupted helices). It has a wide tissue distribution, with its strongest expression being localized to basement membrane zones; thus, it may function to adhere basement membranes to underlying connective tissue stroma.
- KHDRBS3 (also known as Sam68-like phosphotyrosine protein/T-STAR) is an RNA-binding protein that plays a role in the regulation of alternative splicing and influences mRNA splice site selection and exon inclusion, which may play a role as a negative regulator of cell growth, inhibits cell proliferation, is involved in splice site selection of vascular endothelial growth factor (VEGF), and which induces an increased concentration-dependent incorporation of exon in CD44 pre-mRNA by direct binding to purine-rich exonic enhancer.
- C-type lectin superfamily member-2 includes proteins with diverse functions, including cell adhesion, cell–cell signalling, and glycoprotein turnover, which have roles in inflammation and the immune response.
- Apolipoprotein D is a protein closely associated with the enzyme lecithin:cholesterol acyltransferase, an enzyme involved in lipoprotein metabolism.

mRNA was more highly expressed in MSCs than in fibroblasts for the following: tissue factor pathway inhibitor-2, HLA class II histocompatibility antigen DR α chain (MHC-DR-α), MHC-DR-β, MHC-DR-associated protein CD74, and neuroserpin. Proteins encoded by the genes related to these mRNAs are as follows:

- Tissue factor pathway inhibitor-2 is a single-chain polypeptide that can reversibly inhibit the blood clotting protein factor Xa, which is the activated form of the coagulation factor thrombokinase.
- MHC-DR-α is a protein that forms the α subunit of HLA-DR.
- MHC-DR-β, the protein produced from the *HLA-DRB1* gene, called the β chain, attaches to the α chain. Together, they form a functional protein complex called the HLA-DR antigen-binding heterodimer. This complex displays foreign peptides to the immune system to trigger the body's immune response.
- MHC-DR-associated protein CD74 (also known as HLA class II histocompatibility antigen γ chain and as HLA-DR antigens-associated invariant chain or CD74) is a polypeptide involved in the formation and transport of MHC class II protein. The cell surface form of the invariant chain is known as CD74.
- Neuroserpin is a protein secreted from the axons of dorsal root ganglion neurons and is expressed in the late stages of neurogenesis during the process of synapse formation.

Interestingly, these authors used this differential gene expression pattern to define the degree of purity of MSCs that were transplanted in patients undergoing periodontal therapy [51].

A similar approach employing a cDNA microarray of 9600 genes led to the identification of 80 genes that were more than threefold more highly expressed in BM-MSCs than in mature skin fibroblasts [52]. cDNAs highly expressed by MSCs were associated with developmental processes, including embryogenesis, as well as with stem cell function. Strangely, mesenchymal lineage genes were downregulated in MSCs compared with skin fibroblasts.

Another study comparing the gene expression of human AT-MSCs, UCB-MSCs, and BM-MSCs with that of terminally differentiated human fibroblasts showed that 25 and 30 genes, respectively, were found up- and downregulated in MSCs [53]. These included genes that contribute to extracellular matrix (ECM) formation and transcription factors that are mostly involved in collagen and other ECM protein synthesis and degradation. Furthermore, several genes involved in the Wnt pathway were found differentially expressed in MSCs in comparison with fibroblasts. In particular, higher levels of Wnt pathway inhibitors were found in fibroblasts, while in MSCs the downstream activators of the same pathway were found to be upregulated.

Kubo *et al.* used 54 675 DNA microarray probes to analyze the differences in expression levels of undifferentiated MSCs, differentiated MSCs (osteoblasts, chondrocytes, and adipocytes), and fibroblasts [54]; 148 genes were found more than twofold upregulated in MSCs compared with differentiated MSCs or fibroblasts. Furthermore, using qRT-PCR, it was found that the expression level of all 71 genes examined was selectively enhanced compared with differentiated cells and fibroblasts. Among these were genes encoding nine transcriptional factors: *ETV1*, *ETV5*, *FOXP1*, *GATA6*, *HMGA2*, *KLF12*, *PRDM16*, *SIM2*, and *SOX11*. Interestingly, small interfering RNA knockdown of *ETV1*, *ETV5*, *FOXP1*, *GATA6 HMGA2*, *SIM2*, or *SOX11* suppressed the self-renewal capacity of MSCs, whereas those of *FOXP1*, *SOX11*, *ETV1*, *SIM2*, or *PRDM16* reduced their

osteogenic and/or adipogenic differentiation potential. Additionally, the genes for *IL-6*, *VEGF*, *HGF*, and *MET* (the hepatocyte growth factor (HGF) receptor) were highly expressed by undifferentiated MSCs compared with differentiated MSCs and fibroblasts.

Other findings partially corroborate the role of transcriptional factors as genes that discriminate between MSCs and fibroblasts. Sachs *et al.* analyzed the expression of the transcription factors involved in pluripotency – SOX2, OCT-4, and NANOG – in AT-derived MSCs, fibroblasts, and iPSCs generated from fibroblasts [55]. They found that *SOX2* expression level was 10- to 100-fold higher in MSCs than in fibroblasts, but approximately 10-fold lower in MSCs than in iPSCs. *OCT-4* expression was ~10- to 100-fold lower than in iPSCs, but again was significantly higher than in fibroblasts. *NANOG* was also 10- to 100-fold higher in AT-derived MSCs than in fibroblasts and approximately 20-fold lower than in iPSCs.

A wider analysis of the transcriptome profiles of AT-derived MSCs and dermal fibroblasts found 62 genes that were differentially expressed between the two cell populations. Several genes remained expressed after differentiation of AT-derived MSCs and fibroblasts to adipogenic, osteogenic, and chondrogenic lineages: *COL1A1*, *COL1A2*, *EFEMP1*, *FN1*, *GGT5*, and *TPM2* were highly expressed in AT-derived MSCs, while *S100A4* and *TK1* were specific for fibroblasts and fibroblast-derived iPSCs. Proteins encoded by the genes that give rise to the mRNAs listed are collagen type 1 alpha 1, collagen type 1 alpha 2, epidermal growth factor (EGF)-containing fibulin-like ECM protein, fibronectin 1, γ-glutamyltransferase 5, tropomyosin β chain, S100A4 (which has been shown to interact with S100 calcium binding protein A1), and thymidine kinase 1 respectively. It is conceivable that such closely related stromal cells retain "memory" genes able to distinguish both cell populations [56].

An interesting approach to further define the differences between MSCs and fibroblasts may be their microRNA (miRNA) expression profiles. Discovered in early 1990s, these small RNA sequences of approximately 22 nucleotides in length possess regulatory activities, different sequence and expression patterns, and are evolutionarily widespread [57]. Moreover, these noncoding RNA molecules are known to occur in a cell-type-specific manner and to modulate gene expression and/or protein synthesis [58,59]. Recently, Bae *et al.* performed an elegant study to identify genetic elements that can reliably discriminate MSCs from fibroblasts [60]. By comparative gene and miRNA expression profiling analyses, the authors found 64 genes (56 known and 8 uncharacterized) and 21 miRNAs, the expression of which were at least 10-fold and twofold higher respectively in MSCs than in fibroblasts. Approximately one-third of the known genes encoded integral membrane proteins and membrane-associated proteins, suggesting that the cell surface membrane might be a cellular component that is divergent between MSCs and fibroblasts. Additionally, a large number of genes previously associated with cancers were identified as signature genes of MSCs rather than of fibroblasts. Among 21 miRNAs identified, miR-335 was approximately 44-fold higher in MSCs than in fibroblasts. The latter observation is of particular interest since miR-335 expression may also help elucidate some functional differences between MSCs and fibroblasts. Indeed, Tomé *et al.* recently demonstrated that miR-335 expression regulates MSC proliferation, migration, and differentiation [61]. The same authors proposed a working model for miR-335 control and MSC activation. In healthy tissues, Wnt signaling, in coordination with other stimuli, maintains miR-335 expression levels high in MSCs. When tissue damage occurs, interferon-γ (INF-γ) and probably other proinflammatory signals induce miR-335 downregulation, which in turn causes the de-repression of its target genes and leads to "activation" of MSCs by stimulating their proliferative, migratory, and differentiation capacities.

Overall, these findings illustrate that gene profiling has already expanded our knowledge of the molecular specificities of MSCs and fibroblasts. It is likely that new technologies, including next-generation sequencing (NGS), will further define molecular differences between the two cell types. NGS, or second-generation sequencing, also known as massive parallel sequencing or massively parallel sequencing, refers to any of several high-throughput approaches to DNA sequencing using the concept of massively parallel processing. Some of these technologies emerged in 1994–1998 and have been commercially available since 2005. These technologies use miniaturized and parallelized platforms for sequencing of 1 million to 43 billion short reads (50–400 bases each) per instrument run. Many NGS platforms differ in engineering configurations and sequencing chemistry. They share the technical paradigm of massive parallel sequencing via spatially separated, clonally amplified DNA templates or single DNA molecules in a flow cell. This design is very different from that of Sanger sequencing, also known as capillary sequencing or first-generation sequencing, that is based on electrophoretic separation of chain-termination products produced in individual sequencing reactions.

## 30.5 Differentiation potential of mesenchymal stromal cells and fibroblasts

One of the criteria formalized by the International Society for Cellular Therapy in 2006 for the characterization of MSCs was their ability to differentiate *in vitro* into osteoblasts, adipocytes, and chondrocytes [22]. This feature, however, cannot be used to distinguish MSCs and fibroblasts, since, on the one hand, there are reports that fibroblasts are unable to undergo mesenchymal differentiation [52,53,60] but, on the other hand, there are studies that show that fibroblasts do possess the ability to undergo *in vitro* mesenchymal differentiation potential [20,62]. In our own experience, the comparison between AT-derived MSCs and two different commercially available fibroblast cell lines showed no substantial differences in their *in vitro* mesenchymal differentiation capacity [19]. As shown in Figure 30.2, MSCs and

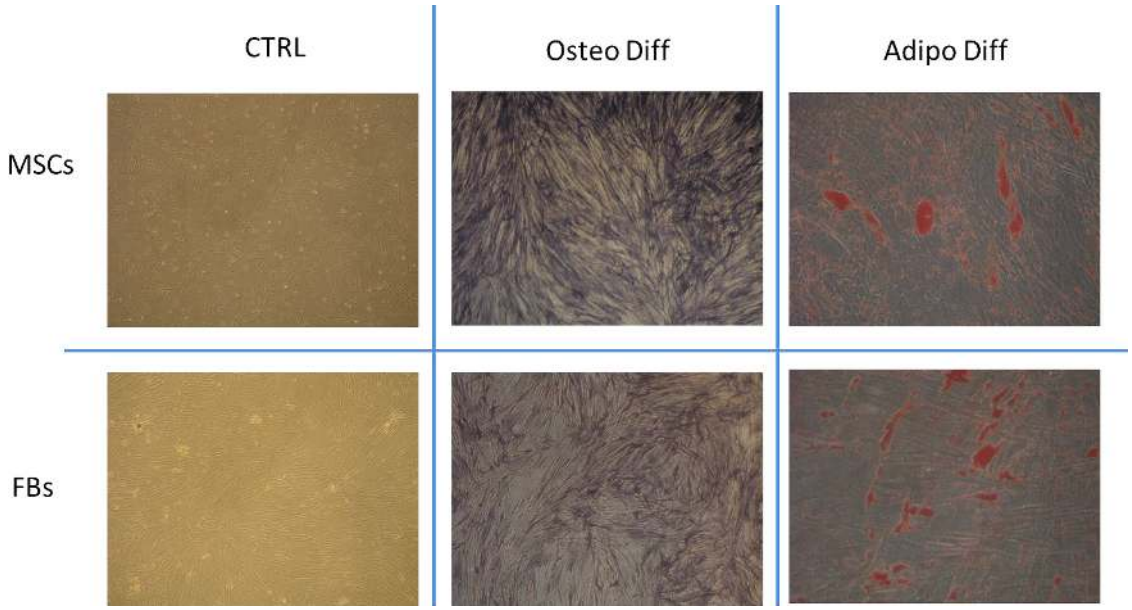



**Figure 30.2** Differentiation potential of MSCs and fibroblasts. Representative photographs of MSCs and fibroblasts assayed for osteogenic and adipogenic differentiation. Left panel: cells grown in control medium. Middle panel: cells cultured in osteogenic medium for 2 weeks were assessed by alkaline phosphatase staining. Right panel: cells cultured in adipogenic medium for 3 weeks were assessed by Oil Red O staining. (See color plate section.)

fibroblasts display comparable capabilities to undergo osteogenic and adipogenic differentiation.

However, to add further complexity to this topic is the discovery that MSC multipotency seems, at least in part, linked to particular MSC subsets. Battula *et al.* sorted two different BM-MSC cell populations by their phenotypic expression of MSCA-$1^+$ $CD56^-$ and MSCA-$1^+$ $CD56^+$ [63]. Interestingly, adipocytes differentiated from MSCs were exclusively derived from the MSCA-$1^+$ $CD56^-$ population, while chondrocytes and pancreatic-like islets were predominantly derived from the MSCA-$1^+$ $CD56^{+/-}$ population. Vaculik *et al.* examined human dermal MSCs using cell surface expression of CD271 and stage-specific embryonic antigen (SSEA) [39]. Adherent dermal cells with increased adipogenic, osteogenic, and chondrogenic potential were enriched in the $CD271^+$ population. The differentiation potential of dermal SSEA-$4^+$ cells, in contrast, appeared to be limited to adipogenesis.

Several aspects of fibroblasts' differentiation potential have also been elucidated. First, it may be related to the age of the cells. Covas *et al.* found that fetal fibroblasts showed a homogeneous capacity to differentiate into adipocytes, chondrocytes and osteocytes, whereas adult foreskin fibroblasts did not differentiate [64]. Contrary to this, another study reported that foreskin fibroblasts exhibited chondrocyte and rare adipocyte and osteocyte differentiation [65].

Hepatocyte differentiation potential of adult human dermal fibroblasts has also been described: in one study, where the *in vitro* and *in vivo* differentiation profile of fibroblasts was comparable to that of mesenchymal-derived hepatocyte-like cells [66].

Another study analyzed the molecular dynamics of differentiation of two closely related stromal cell types, AT-derived MSCs and dermal fibroblasts along adipogenic, osteogenic, and chondrogenic lineages using multiplex RNA-seq technology. They found that undifferentiated donor-matched AT-MSCs and dermal fibroblasts were distinct populations that remained different upon differentiation into adipocytes, osteoblasts, and chondrocytes. The changes in lineage-specific gene expression occurred early in differentiation and persisted over time in both AT-MSCs and dermal fibroblasts. AT-MSCs and dermal fibroblasts exhibited similar dynamics of adipogenic and osteogenic differentiation but different dynamics of chondrogenic differentiation [56].

In conclusion, two facts emerge. First, *in vitro* differentiation along the osteogenic, chondrogenic, and adipogenic lineages is not a useful parameter for distinguishing MSCs from fibroblasts. Second, the evidence for functional differentiation of either MSCs or fibroblasts to cell types outside the mesenchymal lineage is scanty and remains highly controversial.

## 30.6 Immune modulation capability of mesenchymal stromal cells and fibroblasts

The immune system is a very complex cellular and molecular network that requires tight regulation to guarantee a balance between immune surveillance and immune tolerance. Immune modulation by the interaction of a number of cell types and paracrine mechanisms is of great importance. Cell–cell contacts

involved in the immune modulation process include both co-stimulatory and co-inhibitory molecules.

Co-stimulatory molecules include CD80 (B7-1), CD86 (B7-2), CD40, and B7-H4 (B7 homolog 4, also known as V-set domain-containing T-cell activation inhibitor 1). CD80 works in tandem with CD86 to prime T cells.

Co-inhibitory molecules include programmed death-ligand 1 (PD-L1, also known as CD274 or B7 homolog 1). It has been speculated that PD-L1 plays a major role in suppressing the immune system during events such as pregnancy, tissue or organ allografting, and autoimmune diseases. Normally, the immune system reacts to foreign antigens where there is accumulation of the antigen in lymph nodes or spleen, which then triggers a proliferation of antigen-specific $CD8^+$ T cells.

The formation of programmed cell death protein 1 (PD-1) receptor/PD-L1 or B7.1 receptor/PD-L1 ligand complex transmits an inhibitory signal that reduces the proliferation of such $CD8^+$ T cells in the lymph nodes; and supplementary to that, PD-1 is also able to control the accumulation of foreign antigen-specific T cells in the lymph nodes through apoptosis, which is further mediated by a lower regulation of the gene *BCL-2*. B7-H4 is a protein that also belongs to the B7 family of proteins that are expressed on the surface of antigen-presenting cells and interact with ligands such as CD28 on T cells.

In addition, numerous secreted growth factors, cytokines, chemokines, and hormones are also required to promote or inhibit an immune-response. HGF, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 ($PGE_2$), INF-γ, tumor growth factor-beta (TGF-β), interleukin (IL)-1, -6, and -10, and nitric oxide constitute only a small example of biologically active secreted factors involved in immunological responses.

Immune-modulatory properties have been reported for both fibroblasts and MSCs. Fibroblasts have been shown to interact with the immune system as alternative antigen-presenting cells, either activating or downregulating T cells [67,68] and mediating an indirect antiproliferative effects on lymphocytes [69]. A potent effect of fibroblasts on dendritic cell differentiation has also been observed [70].

MSCs were demonstrated to have pleiotropic immune modulatory activities both *in vitro* and *in vivo*, mediated by complex mechanisms that inhibit the function of different immune cell subpopulations of the innate and adaptive immune systems [71].

In general, the immune modulatory effects of MSCs and fibroblasts are mediated by the mechanisms described earlier; namely, cell–cell contact and paracrine activity.

The immune-regulatory role of MSCs and fibroblasts is widely recognized. Haniffa *et al.* compared the immune-suppressive properties of human BM-MSCs and fibroblasts and found that, although both cell populations were equally effective in suppressing T cell proliferation, MSCs were more effective than fibroblasts at inducing T cell cytokine reprogramming [20]. The authors demonstrated that T cells induced INF-γ-dependent IDO expression in fibroblasts, causing a negative-feedback loop that subsequently inhibited T cell proliferation. A molecular mechanism common to fibroblasts and MSCs has been described [72]. Both MSCs and fibroblasts were able to modulate T cell cytokine secretion on stimulation, leading to a marked type 2 helper T cell (Th2) cytokine signature (IL-4 and IL-10), although MSCs appeared more potent in this respect.

Recently, it was demonstrated that indomethacin, a $PGE_2$ inhibitor, affected the immunosuppressive properties of MSCs, but not fibroblasts, on peripheral blood mononuclear cells (PBMCs). When PBMCs were induced to proliferate using the T cell mitogen phytohemaglutinin, both fibroblasts and MSCs inhibited PBMC proliferation. However, indomethacin significantly impaired the inhibitory effect on PBMC proliferation by BM-MSCs but not by fibroblasts [73]. Therefore, it is possible that fibroblasts inhibit PBMC proliferation through a $PGE_2$-independent mechanism. Wada *et al.* confirmed that both MSCs and fibroblasts induce immune suppression, but by different mechanisms. IFN-γ was the key molecule mediating the immunosuppressive activity of fibroblasts [74]. There remains some discrepancy between these findings and those reported by Haniffa *et al.* [20].

These observations could reflect a difference in behavior of MSCs and fibroblasts regarding the activation of immunosuppressive regulatory T cells (Tregs), which are responsible for inhibiting allogeneic T cell proliferation. It was demonstrated that MSCs induced the activation of Tregs through cell-to-cell contact and secretion of soluble factors such $PGE_2$, TGF-β, IL-10, IDO, and galectin [75–77], whereas fibroblasts did not [78,79]. Finally, regarding co-stimulatory molecules, it has been demonstrated that MSCs lack cell surface expression of CD80, CD86, and CD40 [19,80,81]. Likewise, fibroblasts do not express CD80 or CD86 [19,82], but, contrary to MSCs, they do express CD40 [19,65,83]. CD40 binds its ligand CD40L, also known as CD154, which is transiently expressed on T cells and on nonimmune cells under inflammatory conditions. Many molecular and cellular immunological processes are regulated by CD40 engagement, including the initiation and progression of cellular and humoral adaptive immunity [84]. Therefore, the presence of this receptor on fibroblasts may partially explain their different degree of immunosuppressive activity compared with that of MSCs [85]. Skin fibroblasts from patients with the autoimmune disease systemic sclerosis (or scleroderma) had increased levels of CD40 expression [86]. We hypothesize that the lack of CD40 expression on MSCs may help them establish functional immunological activity and help to distinguish MSCs from fibroblasts.

The differences in immune regulation between MSCs and fibroblasts (Figure 30.3) may reflect their behavior in physiological and pathological processes, including homing, inflammation, and fibrosis, where a tight balance between immune stimulatory and immune suppressive stimuli is required.

In summary, further studies are needed to better elucidate the immune modulatory role of different stromal cells populations. To date, the evidence seems to indicate that MSCs possess an enhanced immune suppressive capacity compared with that of fibroblasts.

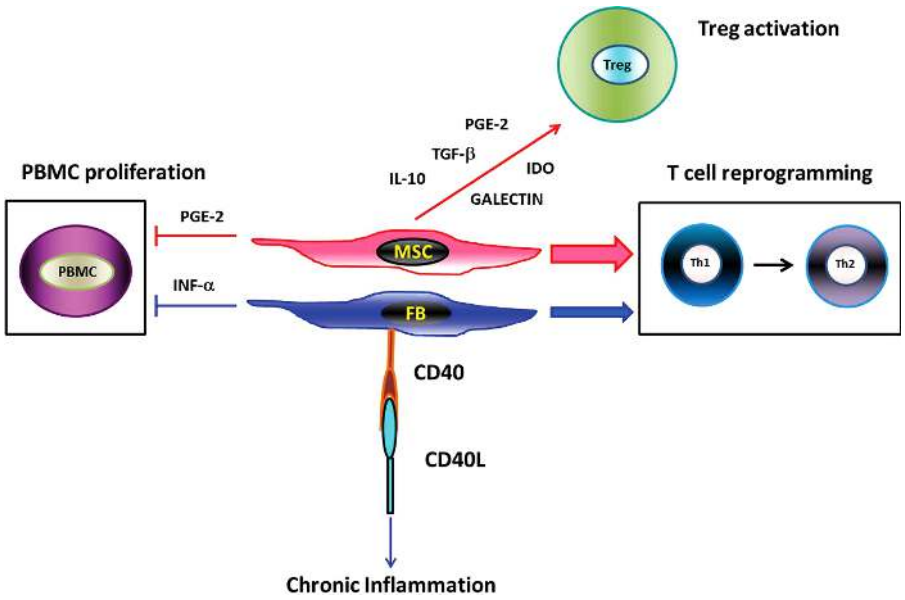



**Figure 30.3** Differences between MSCs and fibroblasts for immune-regulatory properties. Schematic representation of the immune-regulatory properties of MSCs and fibroblasts. Left: MSCs and fibroblasts inhibit PBMC proliferation by distinct mechanisms. Upper: MSCs, but not fibroblasts, induce Treg activation by secreting several factors. Right: MSCs and fibroblasts induce Th cell reprogramming; however, MSCs are twofold more effective than fibroblasts. Lower: fibroblasts, but not MSCs, express the co-stimulatory molecule CD40. CD40–CD40L interaction may lead to chronic inflammation.

## 30.7 Modulation of inflammation by mesenchymal stromal cells and fibroblasts

Strictly related to the immune system, inflammation can be defined as a fundamental defense mechanism aimed at removing harmful stimuli, including damaged cells, irritants, or pathogens, and thus enabling the healing process. Inflammation is a complex process involving sensor and effector cells, expression of surface receptors, and the release of soluble molecules, including cytokines and chemokines. Inflammation has to be tightly regulated in order to achieve a balance between activation and blocking signals. This balance is particularly important, since its dysfunction can lead to the development of chronic inflammation, abnormal pathological tissue remodeling, and autoimmune disorders.

The inflammatory microenvironment includes a complex cell population of both the activators of inflammation comprising macrophages, dendritic cells, and lymphocytes, and the regulating and controlling cells of inflammation comprised of resident fibroblasts, vascular cells, and MSCs, acting mainly by paracrine activity.

Fibroblasts, besides supplying ECM proteins such as collagens and fibronectin, play a role as initiators, modulators, and maintainers of inflammation [87]. During inflammation, fibroblasts are activated and display a proinflammatory phenotype secreting chemokines and cytokines, and acquiring migratory and contractile properties [88–90]. Molecules secreted by activated fibroblasts, such as C–X–C motif chemokine ligand (CXCL)1, CXCL5, IL-8, CXCL12, chemokine (C–C motif) ligand 21, and IL-15, promote the recruitment, survival, and activation of lymphocytes and macrophages. These cells, in turn, augment the activation of fibroblasts, triggering a loop that leads to the enhancement of inflammation. Thus, inhibiting fibroblast activation could reduce inflammation. The presence of proinflammatory signals predisposes to the development of chronic inflammation, which is a pathological condition underlying a wide range of diseases, including myocardial fibrosis after acute myocardial infarction and autoimmune diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease. Consistent reports indicate a preeminent role for fibroblasts in sustaining chronic inflammatory diseases [91,92]. Blockade of the CD40 receptor specifically expressed on fibroblasts, but not MSCs, has been proposed as a potential therapeutic approach to treat chronic inflammation [93].

In contrast to fibroblasts, MSCs have been widely reported to possess anti-inflammatory characteristics [94–96]. It has been demonstrated that, after homing to a site of inflammation (see Chapters 22 and 23), MSCs interact with immune cells, promoting anti-inflammatory pathways [97]. Studies have, in part, elucidated how MSCs exert their anti-inflammatory activity. It has been shown that MSCs are activated by proinflammatory stimuli such as tumor necrosis factor α (TNF-α), secrete $PGE_2$, and upregulate TNF-α-induced protein-6 (TSG-6). $PGE_2$ drives resident macrophages with an M1 proinflammatory phenotype toward an M2 anti-inflammatory phenotype. TSG-6 interacts with CD44 expressed on resident macrophages, decreases toll-like receptor (TLR)2/nuclear factor κ light chain-enhancer of activated B cell signaling, which in turn leads to a reduction of secreted mediators of inflammation [98]. Other mechanisms involving MSCs in controlling macrophage activation have been described [99]: MSCs suppress the generation of mitochondrial reactive oxygen species and inhibit the NOD-like receptor family, pyrin domain containing 3 (NLRP3) inflammasome activation in immune cells by increasing secretion of stanniocalcin-1 in response to activated macrophages [99]. The NLRP3

gene encodes the protein NACHT, LRR, and PYD domains-containing protein (NALP)3 or cryopyrin. This protein interacts with the apoptosis-associated speck-like protein PYCARD/ASC, which contains a caspase recruitment domain, and is a member of the NALP3 inflammasome complex.

An inflammasome is a multiprotein oligomer consisting of caspase 1, PYCARD, NALP, and sometimes caspase 5 (also known as caspase 11 or ICH-3). It is expressed in myeloid cells and is a component of the innate immune system. The exact composition of an inflammasome depends on the activator that initiates inflammasome assembly; for example, dsRNA will trigger one inflammasome composition, whereas asbestos will assemble a different variant. The inflammasome promotes the maturation of the inflammatory cytokines IL-1β and IL-18. The inflammasome is responsible for activation of inflammatory processes and has been shown to induce cell pyroptosis, a process of programmed cell death distinct from apoptosis. During an infection, one of the first forms of defense employed by the innate immune response is a group of pattern recognition receptors encoded in the germline to recognize molecular patterns expressed by invading pathogens. These may either be on the membrane surface (e.g., TLRs and C-type lectin receptors) or inside the cytoplasm (e.g., Nod-like receptors (NLRs) and RIG-I-like receptors). In 2002, a subset of NLRs named NLRP1 were found able to assemble and oligomerize into a common structure that collectively activated the caspase-1 cascade, thereby leading to the production of proinflammatory cytokines, especially IL-1β and IL-18. This NLRP1 multimolecular complex was called the inflammasome and spurred much interest in the following years. Since then, several other inflammasomes have been discovered, two of which are also NLR subsets: NLRP3 and NLRC4. Analogous to the apoptosome, which activates apoptotic cascades, the inflammasome activates an inflammatory cascade. Once active, the inflammasome binds to pro-caspase-1 (the precursor molecule of caspase-1), either homotypically via its own caspase activation and recruitment domain (CARD) or via the CARD of the adaptor protein ASC, which it binds to during inflammasome formation. In its full form, the inflammasome appositions together many p45 pro-caspase-1 molecules, inducing their autocatalytic cleavage into p20 and p10 subunits. Caspase-1 then assembles into its active form consisting of two heterodimers with a p20 and p10 subunit each. Once active, it can then carry out a variety of processes in response to the initial inflammatory signal. These include the proteolytic cleavage of pro-IL-1β at Asp116 into IL1-β, cleavage of pro-IL-18 into IL-18 to induce IFN-γ secretion and natural killer cell activation, cleavage and inactivation of IL-33, DNA fragmentation and cell pore formation, inhibition of glycolytic enzymes, activation of lipid biosynthesis, and secretion of tissue-repair mediators such as pro-IL-1α.

In summary, a review of the literature regarding the role of fibroblasts and MSCs in controlling inflammation shows them to have opposite effects. However, few studies have compared the anti-inflammatory activity of these cells side by side. One of these studies, recently published by our group, analyzed the anti-inflammatory capacity of MSCs derived from AT in comparison with two commercially available fibroblast cell lines [19]. We demonstrated that MSCs, but not dermal fibroblasts, were able to reduce the expression of adhesion molecules and inhibit the levels of inflammatory chemokines RANTES and MCP-1 secreted by the U937 monocytic cell line. Furthermore, we observed that, after priming of both types of stromal cells with TNF-α to mimic the presence of inflammatory stimuli, MSCs increased their inhibitory effect on U937, while fibroblasts increased their proinflammatory characteristics, causing significant augmented release of RANTES and MCP-1 by U937. Other researchers have confirmed our results, using pancreatic islets exposed to inflammatory injury. They demonstrated that MSCs, but not fibroblasts, exerted protective effects. In fact, human islets co-cultured with MSCs and subsequently exposed to a cocktail of proinflammatory cytokines maintained their functionality, as evidenced by glucose-stimulated insulin secretion. This protective effect was not observed in co-cultures with fibroblasts, indicating that the preservation of human islet glucose-stimulated insulin secretion is MSC-dependent [100].

Further data supporting the anti-inflammatory activity of MSCs compared with fibroblasts have been reported by Chen *et al.* [101]. They studied the difference between fibroblasts and MSCs in stimulating the regenerative process and in controlling inflammation in an *in vivo* wound healing model. They examined the differences between MSCs and dermal fibroblasts in releasing paracrine factors. Analysis of MSC- and fibroblast-conditioned media (CM) indicated that the former held greater amounts of cytokines and chemokines involved in wound healing process, such as VEGF, insulin-like growth factor-1, EGF, keratinocyte growth factor, angiopoietin-1 (Ang-1), stromal cell-derived factor-1, macrophage inflammatory protein-1α and -1β, and erythropoietin. In addition, MSC-CM significantly enhanced the migration of macrophages and proliferation and migration of keratinocytes and endothelial cells compared with fibroblast-CM. Moreover, *in vivo* treatment with MSC-CM accelerated wound healing when compared with the administration of fibroblast-CM [101].

Finally, it has been suggested that MSCs control inflammation because they were found to possess an antimicrobial effect whereas fibroblasts did not [102] (and see Chapter 55). In this study, MSCs secreted LL-37, a peptide with a wide array of antimicrobial properties. Conversely, fibroblasts secreted very low levels of LL-37 [102]. The authors speculated that this feature of MSCs might enable them to prevent excess or overexuberant inflammation caused by the presence of bacterial products that occur during wound healing. We wish to emphasize that the processes of scar formation and fibrosis are both linked to inflammation, but are differently regulated by MSCs and fibroblasts. Fibroblasts may negatively regulate these phenomena, probably by depositing excessive amounts of ECM. MSCs, on the other hand, seem to have an antifibrotic activity, probably mediated by anti-inflammatory mechanisms that lead to a

reduction of scar formation following normal wound healing [103] (and see Chapters 58 and 59).

In conclusion, these data demonstrate a clear functional difference between fibroblasts and MSCs as regulators of inflammation. While the former behave as proinflammatory cells, the latter act as anti-inflammatory cells. Although considerable work needs to be done to better understand the mechanisms and molecules involved in such differences, both fibroblasts and MSCs can be considered as sentinel cells in the inflammatory process. Fibroblasts are able to initiate and sustain inflammation when necessary. In contrast, MSCs adopt an anti-inflammatory phenotype and rescue tissues from an excessive inflammatory response. Thus, fibroblasts and MSCs participate synergistically in the inflammatory process to produce a fine balance between pro- and anti-inflammatory stimuli.

## 30.8 Angiogenic properties of mesenchymal stromal cells and fibroblasts

Angiogenesis is the process of new blood vessel formation. Adult angiogenesis usually occurs in the process of tissue regeneration, such as wound healing, and in many pathological situations, such as diabetes mellitus and cancer. Owing to its importance in many pathological processes, much effort has been made to understand the mechanism of neovasculogenesis and, in particular, to evaluate how endothelial cells respond to the angiogenic or anti-angiogenic microenvironment generated during wound healing or by a malignant neoplasm [104].

The interaction of endothelial cells with the microenvironment plays a pivotal role in determining the initiation of angiogenesis, control of progression and maturation of the new vessels. For this reason stromal cells, including fibroblasts and MSCs, and cells of the immune system including macrophages, neutrophils and lymphocytes, have been widely studied in the regulation of endothelial cell activity. In this Chapter we will focus on the involvement of fibroblasts and MSCs in angiogenesis. We will present some comparative studies, showing differences between MSCs and fibroblasts in their contribution to angiogenesis.

There are controversial data in the literature regarding the contribution of fibroblasts to the angiogenic process. Some studies describe a pro-angiogenic effect, while others report an anti-angiogenic effect of fibroblasts [105–108]. Additionally, different populations of fibroblasts have differences in their angiogenic potential [105,109]. In general terms, fibroblasts are able to modulate angiogenesis through a paracrine mechanism and ECM remodeling. Fibroblasts can secrete pro-angiogenic molecules such as VEGF, TGF-β, platelet-derived growth factor, and Ang-1. In addition, fibroblasts produce ECM proteins such as collagens, fibronectin, laminin, and also MMPs, as well as the counteracting tissue inhibitors of metalloproteinases (TIMPs). MMPs have been shown to both enhance and inhibit angiogenesis, including modulation of endothelial cell proliferation, adhesion, and survival, as well as governing tube morphogenesis and regression. ECM remodeling ultimately allows endothelial cell migration, sprouting, and new vessel formation. One study showing a high angiogenic potential of supernatants from fibroblast cultures with an overabundance of proteins associated with ECM constituents [110]. Fibroblasts exert their pro-angiogenic properties by secreting TIMP-1 [111]. Fibroblast-derived matrix proteins are essential for endothelial cell lumen formation as the angiogenic cocktail of Ang-1, angiogenin, HGF, TGF-β, and TNF-α alone failed to form lumens [112].

The pro-angiogenic potential of MSCs has been explored extensively [113–116]. Although a transdifferentiation mechanism was initially proposed as a mechanism by which MSCs mediated angiogenesis, accumulating evidence suggests that MSCs promote angiogenesis by secreting a number of angiogenic factors as well as proteins that regulate endothelial cell motility [117–120]. Additionally, a role has also been ascribed to MSC-derived extracellular vesicles in the *in vitro* and *in vivo* promotion of angiogenesis [121,122].

MSCs also directly interact with endothelial cells to increase tubule numbers, vascular stabilization, and complexity. MSCs are capable of aligning with vessel structure and efficiently stabilize nascent blood vessels *in vivo* by functioning as perivascular precursor cells [123,124]. MSCs are pericytes – they are present in the endothelial wall of all vascularized organs and tissues. Not all pericytes, however, are MSCs: all blood vessels have an endothelium and arteries; arterioles and larger veins have smooth muscle cells in their vessel wall.

Han *et al.* compared the angiogenic activity of MSCs and fibroblasts [125]. They demonstrated that *in vitro* levels of basic fibroblast growth factor were higher in MSCs than in fibroblasts. An *in vivo* model of angiogenesis demonstrated that MSCs induced a significantly higher number of microvessels than fibroblasts did. Similar results were obtained using an excisional wound splinting model in mice: MSCs, but not fibroblasts, significantly enhanced wound healing in both normal and diabetic mice by promoting re-epithelialization, cell infiltration, and angiogenesis. MSC treatment resulted in significantly higher amounts of Ang-1 and VEGF and enhanced capillary density [126].

MSCs, but not fibroblasts, are able to home to perivascular sites in ischemic tissue and promote vascular regeneration. Intriguingly, MSCs aligned along the vessel walls, released pro-angiogenic factors, and augmented microhemodynamics. This led to a reduction of arteriolar vascular resistance and an increase in functional capillary density, thus rescuing the tissue from critical ischemia [127].

In an immunodeficient rat femur nonunion model, human multipotent adipose-derived stromal cells accelerated fracture healing, whereas fibroblasts were not able to do so. In the MSC-treated group this effect was related, at least in part, to a greater capillary density and to an incremental increase in blood flow compared with the fibroblast-treated group. Furthermore,

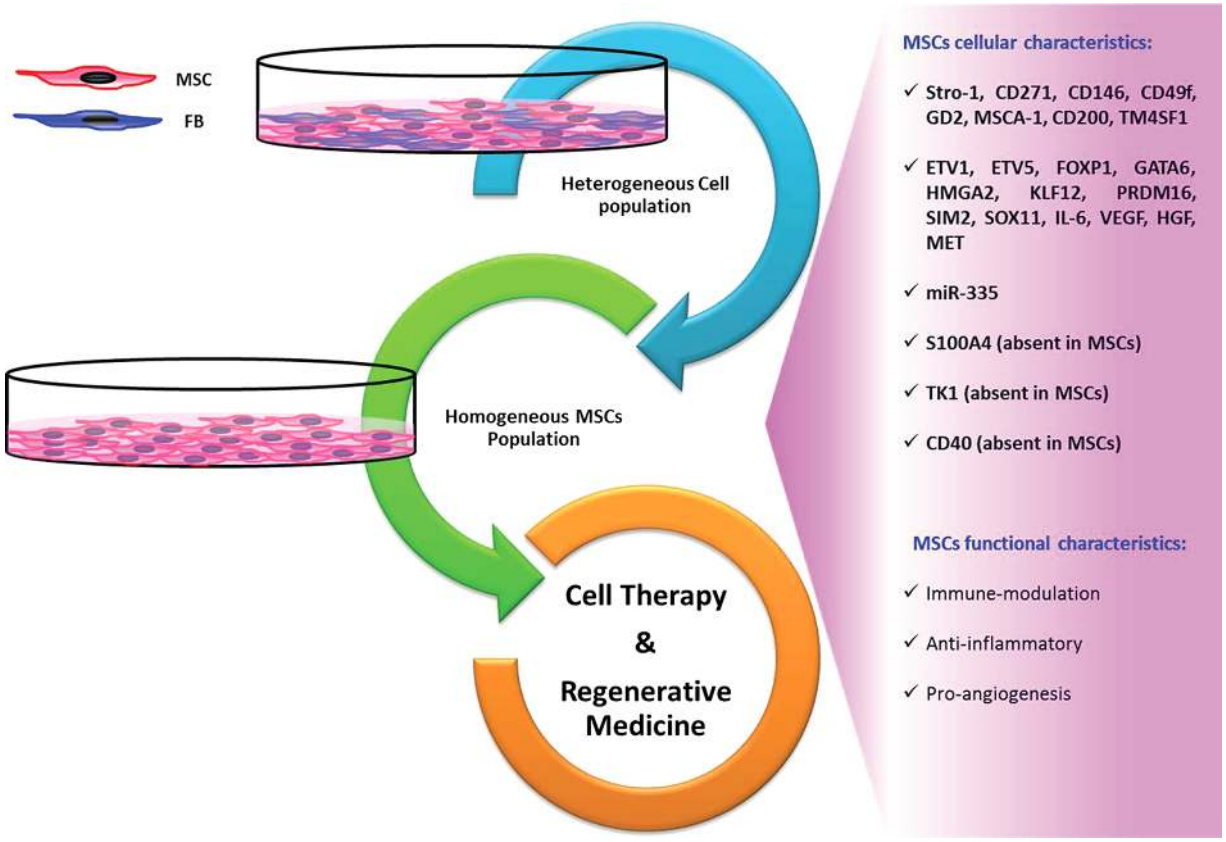



**Figure 30.4** Different molecular markers (genes and miRNAs) as well as surface antigens may be valuable for distinguishing MSCs from fibroblasts.

MSCs, but not fibroblasts, upregulated the expression of the rat pro-angiogenic factors bone morphogenetic protein-2, VEGF, and Ang-1 [128].

In summary, MSCs produce higher levels of pro-angiogenic cytokines than fibroblasts do, as assessed by both mRNA expression and protein [19,125,126]. Extracellular vesicles secreted by MSCs play an important role in angiogenesis promotion [129].

Additionally, MSCs, but not fibroblasts, preferentially home to sites of inflammation [130,131], although a pericytic behavior for fibroblasts has been reported; however, there was a qualitative reduction in vessel coverage by fibroblasts compared with MSCs in this study [132]. Thus, increased angiogenic potential is another parameter for distinguishing MSCs from fibroblasts.

## 30.9 Conclusions

In conclusion, MSCs can be distinguished from fibroblasts by cell surface markers, miRNA activity, immunomodulatory and anti-inflammatory capabilities, and pro-angiogenic activity, but not by the ability to differentiate to osteoblasts, chondrocytes, and adipocytes *in vitro*. These differences are summarized in Figure 30.4.

## References

1 Thomson JA, Itskovitz-Eldor J, Shapiro SS, *et al.* Embryonic stem cell lines derived from human blastocysts. *Science* (1998);**282** (5391):1145–1147.
2 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* (2006);**126**(4):663–676.
3 Noël D, Djouad F, Jorgense C. Regenerative medicine through mesenchymal stem cells for bone and cartilage repair. *Curr Opin Investig Drugs* (2002);**3**(7):1000–1004.
4 Golan-Mashiach M, Dazard JE, Gerecht-Nir S, *et al.* Design principle of gene expression used by human stem cells: implication for pluripotency. *FASEB J* (2005);**19**(1):147–149.
5 Park IH, Zhao R, West JA, *et al.* Reprogramming of human somatic cells to pluripotency with defined factors. *Nature* (2008);**451**(7175): 141–146.
6 Ben-David U, Benvenisty N. The tumorigenicity of human embryonic and induced pluripotent stem cells. *Nat Rev Cancer* (2011); **11**(4):268–277.
7 Fong CY, Gauthaman K, Bongso A. Teratomas from pluripotent stem cells: a clinical hurdle. *J Cell Biochem* (2010);**111**(4):769–781.
8 Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissue. *Transplantation* (1968);**6**:230–247.

9 Sarugaser R, Lickorish D, Baksh D, *et al.* Human umbilical cord perivascular (HUCPV) cells: a source of mesenchymal progenitors. *Stem Cells* (2005);**23**(2):220–229.

10 Alessandri G, Pagano S, Bez A, *et al.* Isolation and culture of human muscle-derived stem cells able to differentiate into myogenic and neurogenic cell lineages. *Lancet* (2004);**364**(9448):1872–1883.

11 Toma JG, McKenzie IA, Bagli D, Miller FD. Isolation and characterization of multipotent skin-derived precursors from human skin. *Stem Cells* (2005);**23**(6):727–737.

12 Seo BM, Miura M, Gronthos S, *et al.* Investigation of multipotent postnatal stem cells from human periodontal ligament. *Lancet* (2004);**364**(9429):149–155.

13 Zuk PA, Zhu M, Ashjian P, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* (2002);**13**(12):4279–4295.

14 Caplan AI, Bruder SP. Mesenchymal stem cells: building blocks for molecular medicine in the 21st century. *Trends Mol Med* (2001); **7**(6):259–264.

15 Caplan AI, Dennis JE. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* (2006);**98**(5):1076–1084.

16 Bernardo ME, Zaffaroni N, Novara F, *et al.* Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* (2007);**67**(19):9142–9149.

17 Delorme B, Charbord P. Culture and characterization of human bone marrow mesenchymal stem cells. *Methods Mol Med* (2007);**140**:67–81.

18 Haniffa MA, Collin MP, Buckley CD, Dazzi F. Mesenchymal stem cells: the fibroblasts' new clothes? *Haematologica* (2009);**94**:258–263.

19 Blasi A, Martino C, Balducci L, *et al.* Dermal fibroblasts display similar phenotypic and differentiation capacity to fat-derived mesenchymal stem cells, but differ in anti-inflammatory and angiogenic potential. *Vasc Cell* (2011);**3**(1):5.

20 Haniffa MA, Wang XN, Holtick U, *et al.* Adult human fibroblasts are potent immunoregulatory cells and functionally equivalent to mesenchymal stem cells. *J Immunol* (2007);**179**(3):1595–1604.

21 Pereira MCL, Secco M, Suzuki DE, *et al.* Contamination of mesenchymal stem cells with fibroblasts accelerates neurodegeneration in an experimental model of Parkinson's disease. *Stem Cell Rev Rep* (2011);**7**:1006–1017.

22 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells: The International Society for Cellular Therapy position statement. *Cytotherapy* (2006);**8**(4):315–317.

23 Bühring HJ, Battula VL, Treml S, *et al.* Novel markers for the prospective isolation of human MSC. *Ann N Y Acad Sci* (2007); **1106**:262–271.

24 Halfon S, Abramov N, Grinblat B, Ginis I. Markers distinguishing mesenchymal stem cells from fibroblasts are downregulated with passaging. *Stem Cells Dev* (2011);**20**(1):53–66.

25 Lv FJ, Tuan RS, Cheung KMC, Leung VYL. Concise review: the surface markers and identity of human mesenchymal stem cells. *Stem Cells* (2014);**32**:1408–1419.

26 Gronthos S, Franklin DM, Leddy HA, *et al.* Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* (2001);**189**(1):54–63.

27 Rosada C, Justesen J, Melsvik D, *et al.* The human umbilical cord blood: a potential source for osteoblast progenitor cells. *Calcif Tissue Int* (2003);**72**(2):135–142.

28 Kuznetsov SA, Mankani MH, Gronthos S, *et al.* Circulating skeletal stem cells. *J Cell Biol* (2001);**153**(5):1133–1140.

29 Chen FG, Zhang WJ, Bi D, *et al.* Clonal analysis of nestin$^-$ vimentin$^+$ multipotent fibroblasts isolated from human dermis. *J Cell Sci* (2007);**120**(Pt 16):2875–2883.

30 Ning H, Lin G, Lue TF, Lin CS. Mesenchymal stem cell marker Stro-1 is a 75 kD endothelial antigen. *Biochem Biophys Res Commun* (2011);**413**(2):353–357.

31 Jones EA, Kinsey SE, English A, *et al.* Isolation and characterization of bone marrow multipotential mesenchymal progenitor cells. *Arthritis Rheum* (2002);**46**(12):3349–3360.

32 Quirici N, Scavullo C, de Girolamo L, *et al.* Anti-L-NGFR and -CD34 monoclonal antibodies identify multipotent mesenchymal stem cells in human adipose tissue. *Stem Cells Dev* (2010);**19**(6): 915–925.

33 Attar A, Ghalyanchi Langeroudi A, Vassaghi A, *et al.* Role of CD271 enrichment in the isolation of mesenchymal stromal cells from umbilical cord blood. *Cell Biol Int* (2013);**37**(9):1010–1015.

34 Wirths S, Malenke E, Kluba T, *et al.* Shared cell surface marker expression in mesenchymal stem cells and adult sarcomas. *Stem Cells Transl Med* (2013);**2**(1):53–60.

35 Buhring HJ, Treml S, Cerabona F, *et al.* Phenotypic characterization of distinct human bone marrow-derived MSC subsets. *Ann N Y Acad Sci* (2009);**1176**:124–134.

36 Schäffler A, Büchler C. Concise review: adipose tissue-derived stromal cells – basic and clinical implications for novel cell-based therapies. *Stem Cells* (2007);**25**(4):818–827.

37 Zhang X, Hirai M, Cantero S, *et al.* Isolation and characterization of mesenchymal stem cells from human umbilical cord blood: reevaluation of critical factors for successful isolation and high ability to proliferate and differentiate to chondrocytes as compared to mesenchymal stem cells from bone marrow and adipose tissue. *J Cell Biochem* (2011);**112**(4):1206–1218.

38 Pilz GA, Ulrich C, Ruh M, *et al.* Human term placenta-derived mesenchymal stromal cells are less prone to osteogenic differentiation than bone marrow-derived mesenchymal stromal cells. *Stem Cells Dev* (2011);**20**(4):635–646.

39 Vaculik C, Schuster C, Bauer W, *et al.* Human dermis harbors distinct mesenchymal stromal cell subsets. *J Invest Dermatol* (2012);**132**(3 Pt 1):563–574.

40 Park JC, Kim JM, Jung IH, *et al.* Isolation and characterization of human periodontal ligament (PDL) stem cells (PDLSCs) from the inflamed tissue: *in vitro* and *in vivo* evaluations. *J Clin Periodontol* (2011);**38**(8):721–731.

41 Lee RH, Seo MJ, Pulin AA, *et al.* The CD34-like protein PODXL and $\alpha_6$-integrin (CD49f) identify early progenitor MSCs with increased clonogenicity and migration to infarcted heart in mice. *Blood* (2009);**113**(4):816–826.

42 Zhidkova OV, Petrov NS, Popov BV. Production and characteristics of the growth and marker properties of mesenchymal stem cells of urinary bladder. *Zh Evol Biokhim Fiziol* (2013);**49**(1): 67–77.

43 Nystedt J, Anderson H, Tikkanen J, *et al.* Cell surface structures influence lung clearance rate of systemically infused mesenchymal stromal cells. *Stem Cells* (2013);**31**(2):317–326.

44 Sarrio D, Franklin CK, Mackay A, *et al.* Epithelial and mesenchymal subpopulations within normal basal breast cell lines exhibit distinct stem cell/progenitor properties. *Stem Cells* (2012);**30**(2):292–303.

45 Martinez C, Hofmann TJ, Marino R, *et al.* Human bone marrow mesenchymal stromal cells express the neural ganglioside GD2: a novel surface marker for the identification of MSCs. *Blood* (2007); **109**:4245–4248.

46 Xu J, Liao W, Gu D, *et al.* Neural ganglioside GD2 identifies a subpopulation of mesenchymal stem cells in umbilical cord. *Cell Physiol Biochem* (2009);**23**(4–6):415–424.

47 Sobiesak M, Sivasubramaniyan K, Hermann C, *et al.* The mesenchymal stem cell antigen MSCA-1 is identical to tissue non-specific alkaline phosphatase. *Stem Cells Dev* (2010);**19**(5):669–677.

48 Delorme B, Ringe J, Gallay N, *et al.* Specific plasma membrane protein phenotype of culture-amplified and native human bone marrow mesenchymal stem cells. *Blood* (2008);**111**(5):2631–2635.

49 Cheong YK, Ngoh ZX, Peh GS, *et al.* Identification of cell surface markers glypican-4 and CD200 that differentiate human corneal endothelium from stromal fibroblasts. *Invest Ophthalmol Vis Sci* (2013);**54**(7):4538–4547.

50 Bae S, Shim SH, Park CW, *et al.* Combined omics analysis identifies transmembrane 4 L6 family member 1 as a surface protein marker specific to human mesenchymal stem cells. *Stem Cells Dev* (2011); **20**(2):197–203.

51 Ishii M, Koike C, Igarashi A, *et al.* Molecular markers distinguish bone marrow mesenchymal stem cells from fibroblasts. *Biochem Biophys Res Commun* (2005);**332**(1):297–303.

52 Brendel C, Kuklick L, Hartmann O, *et al.* Distinct gene expression profile of human mesenchymal stem cells in comparison to skin fibroblasts employing cDNA microarray analysis of 9600 genes. *Gene Expr* (2005);**12**(4–6):245–257.

53 Wagner W, Wein F, Seckinger A, *et al.* Comparative characteristics of mesenchymal stem cells from human bone marrow, adipose tissue, and umbilical cord blood. *Exp Hematol* (2005);**33** (11):1402–1416.

54 Kubo H, Shimizu M, Kawamoto T, *et al.* Identification of mesenchymal stem cell (MSC)-transcription factors by microarray and knockdown analyses, and signature molecule-marked MSC in bone marrow by immunohistochemistry. *Genes Cells* (2009);**14**(3): 407–424.

55 Sachs PC, Francis MP, Zhao M, *et al.* Defining essential stem cell characteristics in adipose-derived stromal cells extracted from distinct anatomical sites. *Cell Tissue Res* (2012);**349**(2):505–515.

56 Jääger K, Islam S, Zajac P, *et al.* RNA-seq analysis reveals different dynamics of differentiation of human dermis- and adipose-derived stromal stem cells. *PLoS ONE* (2012);**7**(6):e38833.

57 Ambros A. MicroRNAs: tiny regulators with great potential. *Cell* (2001);**107**:823–826.

58 Kim VN, Nam JW. Genomics of microRNA. *Trend Genet* (2006); **22**(3):165–173.

59 Bushati N, Cohen SM. MicroRNA functions. *Annu Rev Cell Dev Biol* (2007);**23**:175–205.

60 Bae S, Ahn JH, Park CW, *et al.* Gene and microRNA expression signatures of human mesenchymal stromal cells in comparison to fibroblasts. *Cell Tissue Res* (2009);**335**(3):565–573.

61 Tomé M, López-Romero P, Albo C, *et al.* miR-335 orchestrates cell proliferation, migration and differentiation in human mesenchymal stem cells. *Cell Death Diff* (2011);**18**(6):985–995.

62 Lorenz K, Sicker M, Schmelzer E, *et al.* Multilineage differentiation potential of human dermal skin-derived fibroblasts. *Exp Dermatol* (2008);**17**(11):925–932.

63 Battula VL, Treml S, Bareiss PM, *et al.* Isolation of functionally distinct mesenchymal stem cell subsets using antibodies against CD56, CD271, and mesenchymal stem cell antigen-1. *Haematologica* (2009);**94**(2):173–184.

64 Covas DT, Panepucci RA, Fontes AM, *et al.* Multipotent mesenchymal stromal cells obtained from diverse human tissues share functional properties and gene-expression profile with $CD146^+$ perivascular cells and fibroblasts. *Exp Hematol* (2008);**36**(5): 642–654.

65 Wetzig A, Alaiya A, Al-Alwan M, *et al.* Differential marker expression by cultures rich in mesenchymal stem cells. *BMC Cell Biol* (2013);**14**:54.

66 Lysy PA, Smets F, Sibille C, *et al.* Human skin fibroblasts: From mesodermal to to hepatocyte-like differentiation. *Hepatology* (2007);**46**(5):1574–1585.

67 Kundig TM, Bachmann MF, DiPaolo C, *et al.* Fibroblasts as efficient antigen-presenting cells in lymphoid organs. *Science* (1995); **268**(5215):1343–1347.

68 Shimabukuro Y, Murakami S, Okada H. Interferon-γ-dependent immunosuppressive effects of human gingival fibroblasts. *Immunology* (1992);**76**(2):344–347.

69 Korn JH. Modulation of lymphocyte mitogen responses by cocultured fibroblasts. *Cell Immunol* (1981);**63**(2):374–384.

70 Chomarat P, Banchereau J, Davoust J, Palucka AK. IL-6 switches the differentiation of monocytes from dendritic cells to macrophages. *Nat Immunol* (2000);**1**(6):510–514.

71 Shi M, Liu ZW, Wang FS. Immunomodulatory properties and therapeutic applications of mesenchymal stem cells. *Clin Exp Immunol* (2011);**164**(1):1–8.

72 Krampera M, Cosmi L, Angeli R, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* (2006);**24**(2):386–398.

73 Cappellesso-Fleury S, Puissant-Lubrano B, Apoil PA, *et al.* Human fibroblasts share immunosuppressive properties with bone marrow mesenchymal stem cells. *J Clin Immunol* (2010);**30**(4): 607–619.

74 Wada N, Bartold PM, Gronthos S. Human foreskin fibroblasts exert immunomodulatory properties by a different mechanism to bone marrow stromal/stem cells. *Stem Cells Dev* (2011);**20**(4):647–659.

75 English K, Ryan JM, Tobin L, *et al.* Cell contact, prostaglandin $E_2$ and transforming growth factor β1 play non redundant roles in human mesenchymal stem cell induction of $CD4^+CD25^{High}$ forkhead box $P3^+$ regulatory T cells. *Clin Exp Immunol* (2009); **156**(1):149–160.

76 Zhang Q, Shi S, Liu Y, *et al.* Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. *J Immunol* (2009);**183**(12):7787–7798.

77 Sioud M, Mobergslien A, Boudabous A, Fløisand Y. Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. *Int J Oncol* (2011);**38**(2):385–390.

78 Clark RA, Kupper TS. IL-15 and dermal fibroblasts induce proliferation of natural regulatory T cells isolated from human skin. *Blood* (2007);**109**(1):194–202.

79 Savvatis K, van Linthout S, Miteva K, *et al.* Mesenchymal stromal cells but not cardiac fibroblasts exert beneficial systemic immunomodulatory effects in experimental myocarditis. *PLoS ONE* (2012); **7**(7):e41047.

80 Tse WT, Pendleton JD, Beyer WM, *et al.* Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* (2003);**75**(3):389–397.

81 Najar M, Raicevic G, Fayyad-Kazan H, *et al.* Immune-related antigens, surface molecules and regulatory factors in human-derived mesenchymal stromal cells: the expression and impact of inflammatory priming. *Stem Cell Rev* (2012);**8**(4):1188–1198.

82 Shimabukuro Y, Murakami S, Okada H. Antigen-presenting-cell function of interferon γ-treated human gingival fibroblasts. *J Periodontal Res* (1996);**31**(3):217–228.

83 Fries KM, Sempowski GD, Gaspari AA, *et al.* CD40 expression by human fibroblasts. *Clin Immunol Immunopathol* (1995);**77**(1): 42–51.

84 Elgueta R, Benson MJ, de Vries VC, *et al.* Molecular mechanism and function of CD40/CD40L engagement in the immune system. *Immunol Rev* (2009);**229**(1):152–172.

85 Brouty-Boyé D, Pottin-Clémenceau C, Doucet C, *et al.* Chemokines and CD40 expression in human fibroblasts. *Eur J Immunol* (2000); **30**(3):914–919.

86 Fukasawa C, Kawaguchi Y, Harigai M, *et al.* Increased CD40 expression in skin fibroblasts from patients with systemic sclerosis (SSc): role of CD40–CD154 in the phenotype of SSc fibroblasts. *Eur J Immunol* (2003);**33**(10):2792–2800.

87 Buckley CD, Pilling D, Lord JM, *et al.* Fibroblasts regulate the switch from acute resolving to chronic persistent inflammation. *Trends Immunol* (2001);**22**(4):199–204.

88 Smith RS, Smith TJ, Blieden TM, Phipps RP. Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol* (1997);**151**(2):317–322.

89 Naylor AJ, Filer A, Buckley CD. The role of stromal cells in the persistence of chronic inflammation. *Clin Exp Immunol* (2013); **171**(1):30–35.

90 Enzerink A, Vaheri A. Fibroblast activation in vascular inflammation. *J Thromb Haemost* (2011);**9**(4):619–626.

91 Xia Y, Pauza ME, Feng L, Lo D. RelB regulation of chemokine expression modulates local inflammation. *Am J Pathol* (1997); **151**(2):375–387.

92 Buckley CD. Why does chronic inflammation persist: an unexpected role for fibroblasts. *Immunol Lett* (2011);**138**(1);12–14.

93 Filer A, Raza K, Salmon M, Buckley CD. Targeting stromal cells in chronic inflammation. *Discov Med* (2007);**7**(37);20–26.

94 Iyer SS, Rojas M. Anti-inflammatory effects of mesenchymal stem cells: novel concept for future therapies. *Expert Opin Biol Ther* (2008);**8**(5):569–581.

95 Oh JY, Kim MK, Shin MS, *et al.* The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* (2008);**26** (4):1047–1055.

96 Waterman RS, Morgenweck J, Nossaman BD, *et al.* Anti-inflammatory mesenchymal stem cells (MSC2) attenuate symptoms of painful diabetic peripheral neuropathy. *Stem Cells Transl Med* (2012);**1**(7):557–565.

97 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* (2005);**105**(4): 1815–1822.

98 Prockop DJ. Concise review: two negative feedback loops place mesenchymal stem/stromal cells at the center of early regulators of inflammation. *Stem Cells* (2013);**31**(10):2042–2046.

99 Oh JY, Ko JH, Lee HJ, *et al.* Mesenchymal stem/stromal cells inhibit the NLRP3 inflammasome by decreasing mitochondrial reactive oxygen species. *Stem Cells* (2014);**32**(6):1553–1563.

100 Yeung TY, Seeberger KL, Kin T, *et al.* Human mesenchymal stem cells protect human islet from pro-inflammatory cytokines. *PLoS ONE* (2012);**7**(5):e38139.

101 Chen L, Tredget EE, Wu PYG, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* (2008);**3**(4):e1886.

102 Krasnodembskaya A, Song Y, Fang X, *et al.* Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* (2010);**28**(12): 2229–2238.

103 Abdel Aziz MT, Atta HM, Mahfouz S, *et al.* Therapeutic potential of bone marrow-derived mesenchymal stem cells on experimental liver fibrosis. *Clin Biochem* (2007);**40**(12):893–899.

104 Battegay EJ. Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospects. *J Mol Med (Berl)* (1995);**73**(7): 333–346.

105 Montesano R, Pepper MS, Orci L. Paracrine induction of angiogenesis in vitro by Swiss 3T3 fibroblasts. *J Cell Sci* (1993);**105** (Pt4):1013–1024.

106 Tran-Hung L, Mathieu S, About I. Role of human pulp fibroblasts in angiogenesis. *J Dent Res* (2006);**85**(9):819–823.

107 Souders CA, Bowers SL, Baudino TA. Cardiac fibroblast: the renaissance cell. *Circ Res* (2009);**105**(12):1164–1176.

108 Nehls V, Herrmann R, Hühnken M, Palmetshofer A. Contact-dependent inhibition by cardiac fibroblasts in three-dimensional fibrin gels *in vitro*: implications for microvascular network remodeling and coronary collateral formation. *Cell Tissue Res* (1998);**293** (3):479–488.

109 Sorrell JM, Baber MA, Caplan AI. Human dermal fibroblasts subpopulations; differential interactions with vascular endothelial cells in coculture: nonsoluble factors in the extracellular matrix influence interactions. *Wound Repair Regen* (2008);**16**(2): 300–309.

110 Newman AC, Chou W, Welch-Reardon KM, *et al.* Analysis of stromal cell secretomes reveals a critical role for stromal cell-derived hepatocyte growth factor and fibronectin in angiogenesis. *Arterioscler Thromb Vasc Biol* (2013);**33**(3):513–522.

111 Liu H, Chen B, Lilly B. Fibroblasts potentiate blood vessel formation partially through secreted factor TIMP-1. *Angiogensis* (2008); **11**(3):223–234.

112 Newman AC, Nakatsu MN, Chou W, *et al.* The requirement for fibroblasts in angiogenesis: fibroblast-derived matrix proteins are essential for endothelial cell lumen formation. *Mol Biol Cell* (2011);**22**(20):3791–3800.

113 Gruber R, Kandler B, Holzmann P, *et al.* Bone marrow stromal cells can provide a local environment that favors migration and formation of tubular structures of endothelial cells. *Tissue Eng* (2005); **11**(5–6):896–903.

114 Al-Khaldi A, Al-Sabti H, Galipeau J, Lachapelle K. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. *Ann Thorac Surg* (2003);**75**(1):204–209.

115 Marchionni C, Bonsi L, Alviano F, *et al.* Angiogenic potential of human dental pulp stromal (stem) cells. *Int J Immunopathol Pharmacol* (2009);**22**(3):699–706.

116 Zacharek A, Chen J, Cui X, *et al.* Angiopoietin1/Tie2 and VEGF/Flk1 induced by MSC treatment amplifies angiogenesis and vascular stabilization after stroke. *J Cereb Blood Flow Metab* (2007); **27**(10):1684–1691.

117 Tang YL, Zhao Q, Qin X, *et al.* Paracrine action enhances the effects of autologous mesenchymal stem cell transplantation on vascular regeneration in rat model of myocardial infarction. *Ann Thorac Surg* (2005);**80**(1):229–236, discussion 236–237.

118 De Siena R, Balducci L, Blasi A, *et al.* Omentum-derived stromal cells improve myocardial regeneration in pig post-infarcted heart through a potent paracrine mechanism. *Exp Cell Res* (2010); **316**(11):1804–1815.

119 Phinney DG. Biochemical heterogeneity of mesenchymal stem cell populations: clues to their therapeutic efficacy. *Cell Cycle* (2007); **6**(23):2884–2889.

120 Estrada R, Li N, Sarojini H, *et al.* Secretome from mesenchymal stem cells induces angiogensis via Cyr61. *J Cell Physiol* (2009); **219**(3):563–571.

121 Zhang HC, Liu XB, Huang S, *et al.* Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev* (2012);**21**(18):3289–3297.

122 Bian S, Zhang L, Duan L, *et al.* Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. *J Mol Med (Berl)* (2014); **92**(4):387–397.

123 Sorrell JM, Baber MA, Caplan AI. Influence of adult mesenchymal stem cells on *in vitro* vascular formation. *Tissue Eng Part A* (2009); **15**(7):1751–1761.

124 Au P, Tam J, Fukumura D, Jain RK. Bone marrow-derived mesenchymal stem cells facilitate engineering of long-lasting functional vasculature. *Blood* (2008);**111**(9):4551–4558.

125 Han SK, Chun KW, Gye MS, Kim WK. The effect of human bone marrow stromal cells and dermal fibroblasts on angiogenesis. *Plast Reconstr Surg* (2006);**117**(3):829–835.

126 Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* (2007);**25**(10):2648–2659.

127 Schlosser S, Dennler C, Scweizer R, *et al.* Paracrine effects of mesenchymal stem cells enhance vascular regeneration in ischemic murine skin. *Microvasc Res* (2012);**83**(3):267–275.

128 Shoji T, Ii M, Mifune Y, *et al.* Local transplantation of human multipotent adipose-derived stem cells accelerates fracture healing via enhanced osteogenesis and angiogenesis. *Lab Invest* (2010); **90**(4):637–649.

129 Pascucci L, Alessandri G, Dall'Aglio C, *et al.* Membrane vesicles mediate pro-angiogenic activity of equine adipose-derived mesenchymal stromal cells. *Vet J* (2014);**202**(2):361–366.

130 Karp JM, Leng Teo GS. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* (2009);**4**(3):206–216.

131 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* (2008);**3**(3):301–313.

132 Ghajar CM, Kachgal S, Kniazeva E, *et al.* Mesenchymal cells stimulate capillary morphogenesis via distinct proteolytic mechanisms. *Exp Cell Res* (2010);**316**(5):813–825.

CHAPTER 31

# Derivation of mesenchymal stem/stromal cells from induced pluripotent stem cells

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## 31.1 Introduction

Mesenchymal stem/stromal cells (MSCs) are readily isolated from many tissues and capable of extensive *ex vivo* expansion [1,2]. MSCs can typically be differentiated into cells of the mesodermal lineage, while some investigators have also reported MSC differentiation into cells of ectodermal lineage [3,4]. MSCs have low immunogenicity and can be transplanted across major histocompatibility complex (MHC) barriers, making them candidates for both patient-specific and "off-the-shelf" tissue regeneration therapies [5]. Administration of MSCs is associated with very little toxicity, perhaps in part owing to their anti-inflammatory and immunomodulatory properties [6,7]. Understanding the effects of the age, quality, and quantity of *ex vivo* expanded MSCs is of relevance to their potential future widespread clinic use [6].

The doses of MSC required for human transplantations can be as high as 6 billion cells. Potential targets for their use include modulation of organ transplant rejection (see Chapter 56), autoimmune disease (see Chapter 54), regeneration of bone and cartilage (Chapter 47), repair of cardiac muscle (Chapter 45), inflammatory bowel disease (Chapter 50), and neurological disorders (Chapters 48 and 49) [6,8,9]. However, as people age, bone marrow MSCs *in vivo* decline in quantity and quality [10]. MSCs harvested from adult bone marrow comprise only around 0.001–0.01% of total bone marrow mononuclear cells and proliferate only for a short duration [11]. This decline is disadvantageous in the elderly, who are the population most in need of tissue repair therapies. There is thus a major unmet

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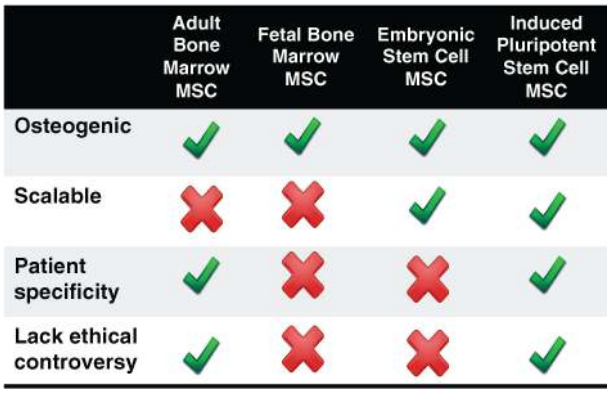



**Figure 31.1** Summary of the positive and negative aspects of different sources of MSCs for future regenerative medicine applications.

requirement for either a technology to improve the current properties of adult MSCs or to develop renewable, superior sources of MSCs.

MSC have been isolated from almost every organ in the mouse and human [1,12,13] with usually minor "organ-specific" or "niche" differences reported [14–19]. For example, bone marrow MSCs are excellent at forming bone, cartilage, and adipose tissue *in vitro* but have a short-term *ex vivo* proliferative capacity, whereas MSCs from term placenta are poor at adipose differentiation *in vitro* but have superior long-term proliferation [18]. Thus, MSCs from different organs may be suited for different clinical purposes, and this must be clearly elucidated prior to their clinical use [6,20].

## 31.2 Mesenchymal stem/stromal cells as candidates for cellular therapy

There is a major unmet need for a robust source of osteogenic stem cells that could be patient specific or, if derived from an unrelated MHC-unmatched donor, used as an "off the shelf" cellular therapy for nonuniting bone fractures (see Chapter 47). This has instigated the search for a source of stem or progenitor cells that could be used in conjunction with synthetic bone graft materials as a treatment to optimize fracture healing [21] (Figure 31.1).

## 31.3 Mesenchymal stem/stromal cells

MSCs are multipotent cells with the ability to differentiate into cells of the mesenchymal lineage [11,22], rendering them a potentially ideal cell source for use in a range of clinical applications, including bone repair [23,24]. MSCs were originally described as originating from bone marrow [25], but they have subsequently been isolated from adipose tissue [26], umbilical cord blood [27], peripheral blood [28], skeletal muscle [29], placenta [18], dental tissue [30], fetal tissue [31], and synovial fluid [32]. Owing to the tissue distribution of MSCs and variation in isolation methods, MSCs derived from the primary tissue result in a heterogeneous population of cells, leading to inconsistencies in the characteristics of MSCs with no specific cell surface marker to unequivocally identify them.

For these reasons the International Society for Cellular Therapy (ISCT) proposed minimal criteria for the classification of adult bone marrow MSCs [33,34]. These criteria were (1) MSCs must display a fibroblast-like morphology, (2) be plastic-adherent *in vitro*, (3) be able to undergo *in vitro* mesodermal differentiation into osteocytes, adipocytes, and chondrocytes, and (4) must express a panel of markers characteristic of, but not unique to, MSCs, including CD105, CD73, and CD90, as well as lacking expression of hematopoietic and endothelial markers such as CD45, CD11b, and CD31 [33,34].

This area took another significant step forward with the publication in 2013 of a joint statement from the International Federation for Adipose Therapeutics and Science and the ISCT on stromal cells from the adipose tissue-derived stromal vascular fraction and culture-expanded adipose tissue-derived stromal/stem cells (see Chapter 1).

The centrifuged but uncultured stromal vascular fraction was identified as being $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$ (platelet endothelial cell adhesion molecule), and $CD34^+$, while cell surface positivity for CD13 (alanine aminopeptidase), CD73, CD90, and CD105 were best used in conjunction with CD45, CD235a, and CD31.

*Ex vivo*-expanded adipose tissue-derived stromal/stem cells retain cell surface markers expressed by other MSCs, including CD44 (the hyaluronic acid receptor), CD73, CD90, and CD105 and are also negative for CD45 and CD31, but can be distinguished from bone-marrow-derived MSCs by being $CD36^+$ (fatty acid translocase, also known as glycoprotein IIIb) and $CD106^-$ (vascular cell-adhesion molecule 1).

## 31.4 Adult bone-marrow-derived mesenchymal stem/stromal cells

Adult bone marrow-derived MSCs have been used clinically in the treatment of fractures [23]. However, they are limited by their rarity, the associated invasive harvesting technique required and their finite proliferative capacity in *ex vivo* expansion cultures, which is a necessary for obtaining enough cells for clinical translation [35].

## 31.5 Fetal tissue-derived mesenchymal stem/stromal cells

Another potential source of MSCs for bone repair is fetal bone marrow tissue [2,31]. These cells have been shown to possess ontological advantages over MSCs from adult tissues, with fetal bone-marrow-derived MSCs possessing more primitive

characteristics, such as faster proliferation and higher stromal precursor antigen-1 expression, indicative of colony forming and differentiative capacity [31]. Fetal bone-marrow-derived MSCs have also been shown to produce greater osteogenic matrix deposition *in vitro* and *in vivo* compared with adult bone-marrow-derived MSCs [17,36]. Despite these properties, fetal bone-marrow-derived MSCs are not scalable because of the paucity of source material and their isolation entails tissues being derived from aborted fetuses.

## 31.6 Embryonic stem cells

Human embryonic stem cells (ESCs) hold promise for regenerative medicine and tissue engineering applications. They provide a potentially unlimited source of pluripotent cells capable of undergoing differentiation into any cell type [37,38]. Human ESCs are characterized by the expression of the nuclear transcription factors *Oct4*, Nanog, and *SOX2* and the surface markers Tra-1-60, Tra-1-81, stage-specific embryonic antigen (SSEA)3, and SSEA4. However, undifferentiated ESCs form teratomas *in vivo*. This has hindered their clinical use, although a search of ClinicalTrials.gov in August 2015 listed five clinical trials using them or their differentiated progeny. An alternative approach would be to differentiate ESCs to committed progenitor cells, capable of expansion and differentiation to the desired mature cell type under environmental or niche influences, while negating the possibility of tumor formation *in vivo*.

## 31.7 Embryonic stem-cell-derived mesenchymal stem/stromal cells

To date, a number of studies have differentiated ESCs into MSCs using one or more of four methods. These are (1) immortalization, (2) co-culture with mouse MSCs, (3) epitope selection, or (4) physical selection [39–43]. However, these methods are not ideal for clinical translation, owing to the requirement of genetic modification, the use of xenogeneic tissues (mouse feeder cell layer), low throughput, labor intensity, or multiple culture steps.

A standard method of differentiating ESCs into MSCs involves an intermediate stage using embryoid body (EB) formation, an *in vitro* replication of early embryonic development [44], followed by physical selection of the cell type of interest. Undifferentiated ESCs are cultured as EBs for 10 days, at which point the cells are transferred to an extracellular-matrix-coated tissue culture plate. After further culture the outgrowth cells are physically selected and cultured in MSC growth medium [44,45]. The resulting cells expressed standard MSC markers, lacked expression of hematopoietic markers, and displayed greater *in vitro* and *in vivo* osteogenicity than that of adult bone-marrow-derived MSCs [44]. Unfortunately, a heterogeneous population of cells results from EB differentiation, rendering this physical selection method undesirable.

More recently, methods have been developed that provide feeder-free, sorting-free, and EB-free alternatives [37,46]. For example, Boyd *et al*. developed a method for differentiating human ESCs into a monolayer by culturing them in a commercial endothelial growth medium [37]. After 30–40 days the cells had differentiated into an epithelial monolayer, and with subsequent passaging under MSC culture conditions the cells further differentiated into MSCs. The resulting cells showed the characteristic MSC surface phenotype and displayed *in vitro* osteogenic and chondrogenic, but not adipogenic, differentiation.

Owing to the change in cell morphology it was hypothesized that the cells were undergoing epithelial-to-mesenchymal transition (EMT). However, the classic E-cadherin to N-cadherin switching mechanism, observed with definitive EMT [47], was not observed, as E-cadherin was present at all stages of differentiation. Although other mesodermal genes, such as *Gata4*, *Runx1*, and *BMP4*, were induced during the differentiation process, an increase in N-cadherin was not examined. However, this technique is scalable, uses defined medium to induce ESC to MSC differentiation, and avoids clinically untranslatable methods of co-culture, physical selection, and epitope selection. It is a very low throughput and time-consuming process, which limits its clinical applicability.

## 31.8 Induced pluripotent stem cells

In 2006, Yamanaka's group in Japan discovered that normal murine adult cells could be reprogrammed in the laboratory and converted into pluripotent ESC-like cells capable of unlimited proliferation and differentiation [48]. This was done by transducing terminally differentiated adult somatic cells such as fibroblasts with the four genes *Oct3/4*, *SOX2*, *Klf4* and *c-Myc*. This revolutionized the field of cell and developmental biology, with significant implications for regenerative medicine and tissue engineering.

In 2007, Thomson and colleagues demonstrated reprogramming of somatic cells into induced pluripotent stem cells (iPSCs) by transducing adult cells with a different quartet of four reprogramming factors: *Oct4*, *SOX2*, *Nanog*, and *LIN28* [49]. The iPSCs were morphologically similar to ESCs, were karyotypically normal, expressed telomerase activity, indicating their indefinite proliferative ability, and expressed cell surface markers and proteins characteristic of human ESCs: *SSEA3*, *SSEA4*, *Tra-1-60*, *Tra-1-80*, *Oct4*, *SOX2*, and *Nanog*. Like ESCs, these iPSCs had the capacity to differentiate into cells of all three primary germ layers in a teratoma assay [49].

Initially, iPSCs were derived using viral transduction reprogramming technology, which is clinically untranslatable [48–50]. However, with rapid advances in reprogramming technology, iPSC lines have been generated using episomal (nonviral) vectors, chemicals, and soluble proteins, which are more suitable for clinical translation [51].

The generation of patient-specific iPSCs obviates the need to harvest tissues enmeshed in ethical or controversial issues, such as embryonic or fetal sources. The starting material can potentially be any tissue in the adult body. iPSCs would be a scalable source of patient-specific stem cells, rendering them an ideal future source to exploit for tissue repair applications.

## 31.9 Small-molecule methods for differentiating pluripotent stem cells into mesenchymal stem/stromal cells

Watabe *et al.* developed a method to differentiate human ESCs into an epithelial monolayer using the small-molecule inhibitor SB431542 [52]. Recently, it has been established that Activin/Nodal signaling can block differentiation of ESCs, suggesting that members of the transforming growth factor-β (TGF-β) family are involved in cell fate decisions of ESCs and in the maintenance of pluripotency (Figure 31.2) [55].

Nodal acts by binding heteromeric complexes between type I (ALK4 and -7) and type II (ActRIIB) Activin receptors, which in turn act through the Smad2/Smad3 signaling pathway (Table 31.1). Nodal and Activin share type I and II receptors (ALK4, ActRIIB) and have the same Smad signaling pathway (Smad2/Smad3) [56,57]. Thus, a competitive inhibitor of the ATP binding site of ALK4/5/7 was used to induce differentiation

**Table 31.1** Signaling pathway of Activin and selected members of the TGF-β superfamily blocked by SB431542.

| Ligand | Receptor | | Smad Pathway |
|---|---|---|---|
| | Type II | Type I | |
| Activin A | ActRII | ALK4 (ActRIB) | Smad2 |
| Activin B | ActRIIB | ALK7 | Smad3 |
| Inhibin A | ActRII | ALK7 | Smad3 |
| Inhibin B | ActRIIB | ALK7 | Smad3 |
| BMP-2 | ActRII | ALK2 | Smad1 |
| BMP-4 | ActRIIB | ALK3 | Smad5 |
| BMP-7 | BMPRII | ALK6 | Smad8 |
| TGF-β1 | TGFβRII | ALK5 | Smad2 |
| TGF-β2 | TGFβRII | ALK1 | Smad3 |
| TGF-β3 | TGFβRII | ALK1 | Smad3 |
| Myostatin | ActRII | ALK4 | Smad2 |
| | ActRIIB | ALK5 | Smad3 |
| Nodal | ActRII | ALK4 | Smad2 |
| | ActRIIB | ALK7 | Smad3 |

Adapted from [53].
BMP: bone morphogenetic protein; ActR: Activin receptor kinase; ALK: activin-like kinase; ATP: adenosine triphosphate.

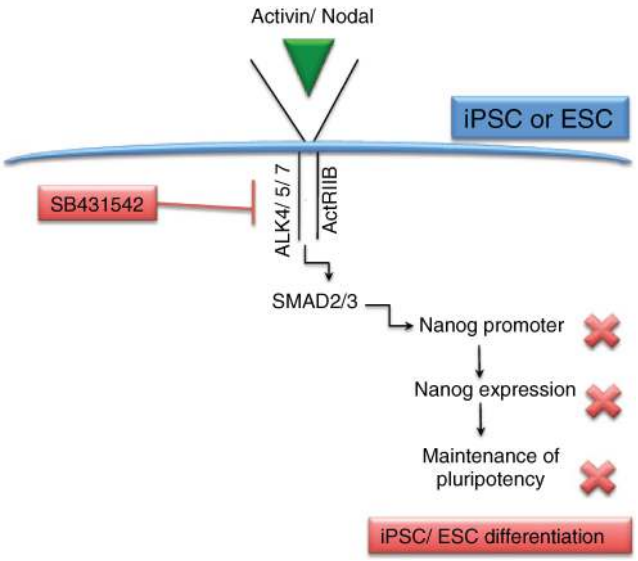



**Figure 31.2** Overview of the antagonism of Activin/Nodal-activated signaling pathways by SB431542. Cascades of downstream targets in the Activin/Nodal signaling pathway are shown. The inhibitory effects of the small-molecule inhibitor SB431542 are also indicated. Binding of heteromeric complexes between type I (ALK4/5/7) and type II (ActRIIB) Activin receptor is activated by ligand binding and signals through the Smad2/Smad3 pathway. The small-molecule inhibitor SB431542 acts as a competitive inhibitor of the ATP-binding site of ALK4/5/7 and is known to have the effect of inducing differentiation of human ESCs. *Source*: adapted from Harrison 2005 [53] and Vallier 2009 [54].

of human ESCs [55,58]. The application of the small molecule inhibitor SB431542 was shown to promote differentiation by inhibiting Nanog promoter activity and thus downregulating other genes responsible for maintaining pluripotency [53,58,59].

SB431542 has also been used in conjunction with the standard EB method to enhance differentiation of human ESCs, applying it to the outgrowth cells from the center of the attached EB [60]. The resulting cells were shown to upregulate genes associated with myogenic development and function and showed contraction ability *in vivo*. The cells also expressed markers characteristic of MSCs with mesodermal differentiation ability. Despite this advance, the methodology utilized still involves physical selection and the formation of the intermediate EB structure, again limiting its clinical applicability.

## 31.10 Derivation of induced pluripotent stem cell–mesenchymal stem/stromal cells through a novel transforming growth factor-β inhibitor method

As previously outlined, a sorting-free, EB-free monolayer method has been developed to differentiate ESCs into an epithelial monolayer and further into MSCs [37]. This is a desirable method as it avoids clinically untranslatable issues of co-culture

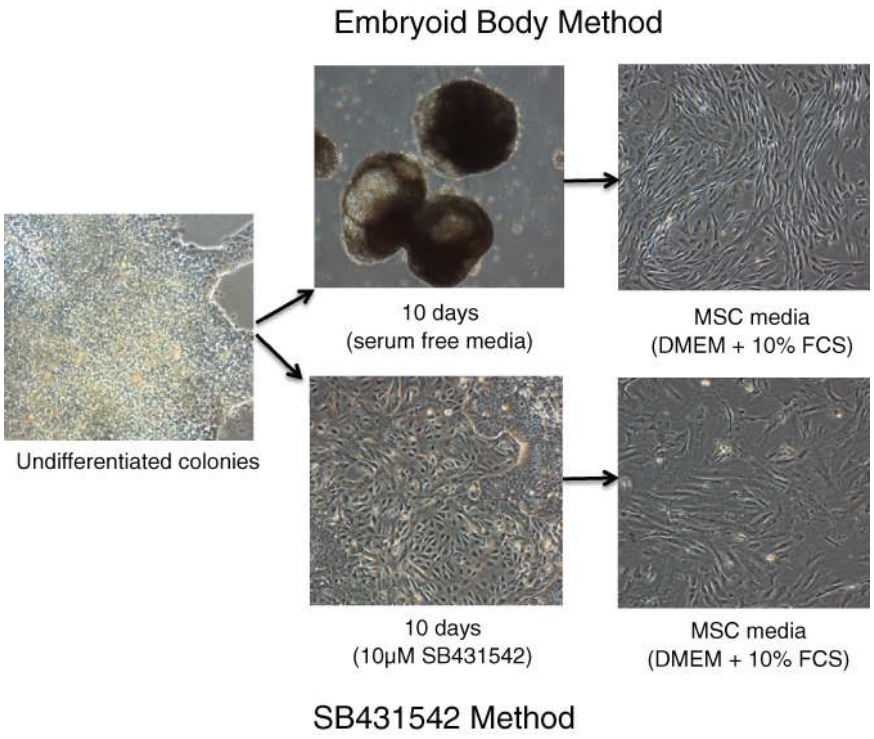



**Figure 31.3** Experimental outline for differentiating iPSCs to MSCs. To differentiate iPSCs to MSCs two methods were utilized. The first was an EB method, which involves culturing undifferentiated iPSC colonies (upper panel) in suspension for 10 days in noncoated dishes until EBs develop (upper left). The EBs are then transferred to a standard tissue culture flask in MSC medium consisting of Dulbecco's modified Eagle medium (DMEM) plus 10% fetal calf serum (FCS). After adhering to the plastic, the differentiated MSC-like cells grow out from the EB and are further cultured in MSC medium (upper right). The second method involved adding a small molecule SB431542, which is an inhibitor of the TGF-β pathway, each day for 10 days to the iPSCs (lower panel). The resulting cells display an epithelial-like morphology (lower left) and when subsequently cultured in MSC medium the cells differentiate into cells with a fibroblast-like morphology (lower right). (See color plate section.)

and physical and epitope selection, but does involve a prolonged differentiation period.

Our laboratory developed a novel method combining the methods of Watabe *et al.* [52] and Boyd *et al.* [37] using the SB431542 inhibitor. This procedure has been shown to induce differentiation of pluripotent stem cells into a uniform epithelial monolayer in only 10 days [61]. Subsequent culture in MSC medium resulted in fibroblast-like cells that displayed MSC markers, lacked pluripotency markers, and were capable of undergoing mesodermal differentiation. In a direct comparison we found that this method was more rapid and less likely to produce non-mesodermal cells than the standard EB method (Figure 31.3), even though similar MSC-like cells were produced.

Through analyzing the morphological changes occurring during the differentiation of MSCs from iPSCs and ESCs using the inhibitor method, it was evident that the resulting MSC-like cells formed a homogeneous monolayer. This finding enhances the clinical amenability of this method for iPSC–MSC derivation, as it negates the need for the physical selection of desired cell types, which is a requirement of the standard EB method and which was used as a comparison method in this study. The application of the TGF-β pathway inhibitor, SB431542, blocked maintenance of pluripotency by preventing activation of the Smad2/Smad3 signaling pathway and subsequent Nanog promoter activity. This resulted in inhibition of pluripotency, inducing differentiation of iPSCs through an EMT-like process. iPSCs and ESCs differentiated into homogeneous monolayers of epithelial-like cells expressing E-cadherin, which when cultured further in fetal MSC medium differentiated into MSC-like cells with a decrease in E-cadherin but an increase in N-cadherin. The observed E-cadherin to N-cadherin expression switching has been documented in definitive EMT [47]. This mechanism was supported by the fact that, during the course of our initial study, two independent studies found that a reverse mechanism, involving mesenchymal-to-epithelial-like transition is, indeed, crucial in the initiation phase of the reprogramming of fibroblasts to iPSCs [62,63] (Figure 31.4).

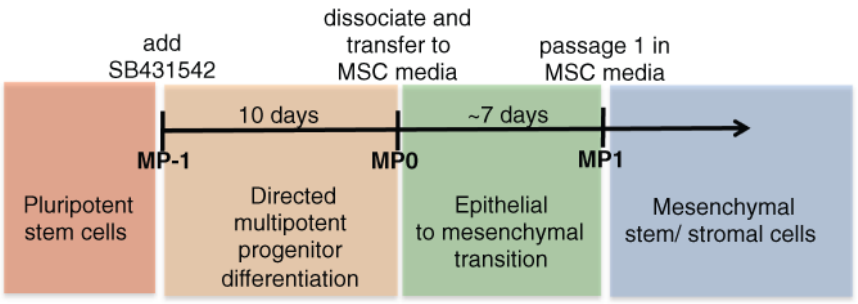



**Figure 31.4** A timeline of the proposed EMT occurring during the differentiation of pluripotent stem cells into MSCs. The differently shaded areas represent discrete steps of differentiation during SB431542 treatment and differentiation into MSCs. MP: mesenchymal passage.

Thus, MSC-like cells were derived from iPSCs using a sorting-free, EB-free monolayer method, circumventing the use of clinically untranslatable methods such as co-culture, physical selection, epitope selection, and viral transduction. Furthermore, the derivation required a rapid 10-day incubation period with SB431542 before differentiation was induced, unlike other methods requiring 40 days incubation [37]. We therefore believe that this method could be employed for the large-scale manufacturing of pluripotent stem-cell-derived MSCs, free of ethical concerns, for clinical use.

## 31.11 Mesenchymal characterization of induced pluripotent stem cell–mesenchymal stem/stromal cells derived through the inhibitor method

The various sources of MSCs used in this study were subjected to identical culture conditions in the same laboratory to control for the differences that occur when samples are processed in different laboratories. The fetal bone-marrow-derived MSCs, iPSC–MSC-derived MSCs (inhibitor and EB), and, where appropriate, ESC-derived MSCs (by both the inhibitor and EB methods) were fully characterized in accordance with the criteria of the ISCT for the classification of MSCs [33]. They were all found to be adherent fibroblast-like cells, possessing mesodermal differentiative capacity under permissive conditions, expressing various MSC markers and adhesion molecules, and lacking hematopoietic and endothelial markers.

The iPSC–MSCs derived through the novel inhibitor method expressed an immunophenotype similar to that of fetal bone-marrow-derived MSCs with positive expression of the markers CD73, CD90, and CD105 used to identify MSCs according to the ISCT criteria [33] at equivalent levels to those of fetal bone-marrow-derived MSCs. The iPSC–MSCs also lacked expression of hematopoietic, macrophage, and monocyte markers (CD11b, CD14, human leukocyte antigen (HLA)-DR, CD45, and CD34), again consistent with the ISCT criteria [33]. Other markers commonly expressed by MSCs were also expressed by iPSC–MSCs (inhibitor), including CD29, CD13, CD44, and CD146. Interestingly, iPSC–MSCs (EB) lacked expression of the fibroblast marker (CD13), which was positively expressed by iPSC–MSCs (inhibitor) and fetal bone-marrow-derived MSCs, suggesting the novel inhibitor method may facilitate the derivation of MSCs more similar to fetal bone-marrow-derived MSCs than to adult MSCs.

Immunofluorescence was used assess the loss of pluripotency markers expressed by iPSCs and ESCs as differentiation proceeded. Fetal bone-marrow-derived MSCs and iPSC–MSCs (inhibitor and EB) lacked expression of pluripotency markers such as *Oct4*, Nanog, Stella, SSEA3, Tra-1-60 and Tra-1-81. For the future therapeutic use of these cells it is critical that they are fully differentiated and do not express markers associated with pluripotency, and thus tumorigenicity. During the differentiation from iPSCs to iPSC–MSCs (inhibitor), cells gained expression of mesodermal markers, including vimentin, confirming that the iPSCs had undergone differentiation into MSCs using the inhibitor method. Interestingly, SSEA4 was expressed by undifferentiated iPSCs and iPSC–MSCs at mesenchymal passage 2 after differentiating through the inhibitor method. Although considered a typically archetypal pluripotent cell marker, SSEA4 expression by adult MSC populations has been documented by others [64].

The *in vitro* mesodermal differentiative capacity of iPSC–MSCs (inhibitor) was found to be comparable to that of fetal bone-marrow-derived MSCs, with both sources of MSCs readily undergoing osteogenic, adipogenic, and chondrogenic differentiation after 28 days' culture in permissive conditions. The ESC–MSCs derived through the novel inhibitor method displayed greater osteogenic differentiation than those derived through the standard EB method, as visualized by von Kossa and Alizarin red S staining. This increase in osteogenic differentiative ability supports the use of this method over the standard EB method for deriving MSCs with superior osteogenic capacity for clinical use for facilitating the healing of bone fractures.

## 31.12 Immune tolerance to induced pluripotent stem cell–mesenchymal stem/stromal cells

Another finding that would render the use of these cells clinically advantageous was the fact that iPSC–MSCs (inhibitor and EB methods) were found to express low levels of HLA-ABC and completely lacked expression of HLA-DR. This pattern of expression is consistent with that of fetal bone-marrow-derived MSCs. This could be indicative of low immunogenicity of iPSC–MSCs *in vivo*, similar to that of fetal bone-marrow-derived MSCs [65], which would be useful for clinical application.

## 31.13 Kinetics of the proliferation of induced pluripotent stem cell–mesenchymal stem/stromal cells

The iPSC–MSCs (inhibitor) and iPSC–MSCs (EB) did not proliferate as rapidly as fetal bone-marrow-derived MSCs. However, their population doubling time indicates their suitability for scale-up, as is required for clinical use. The growth kinetics of fetal bone-marrow-derived MSCs was in agreement with other studies of fetal bone-marrow-derived MSCs, confirming their rapid cumulative population doublings [36]. The growth kinetics of iPSC–MSCs (inhibitor) indicated that the cumulative population doublings were comparable to those of adult MSCs as described in literature [31,36]. The kinetics of proliferation of iPSC–MSCs were found to be comparable to those of ESC-derived MSCs [43]. Thus, the finding that these kinetics were

slower than those of fetal bone-marrow-derived MSCs was not surprising.

## 31.14 Tumorigenic potential of induced pluripotent stem cell–mesenchymal stem/stromal cells

The clinical use of MSCs derived from pluripotent stem cells such as ESCs and iPSCs has been limited by the fact that undifferentiated cells (iPSCs and ESCs) are known to form teratomas *in vivo* [49,66]. Thus, to explore the safety of using these cells in future clinical applications, iPSC–MSCs (inhibitor and EB) were injected intramuscularly into immunosuppressed mice and monitored for tumor formation at the injection site. Three mice were injected per cell type and it was expected that for undifferentiated stem cells one teratoma would form in every two mice injected [49]. We found that two out of the three NOD/SCID mice injected with iPSCs did form teratomas. Hematoxylin and esosin staining showed the presence of cells derived from all three primitive germ layers [49].

Since recent studies have focused attention on identifying and classifying chromosomal aberrations that have the potential to affect the safe use of these cells in future clinical applications [67], chromosomal stability was investigated by karyotyping. All samples tested, including fetal bone-marrow-derived MSCs, iPSC–MSCs (inhibitor), and iPSC–MSCs (EB), were found to be karyotypically normal.

**Table 31.2** MSC and pluripotency markers used to characterize iPSC–MSCs.

| Markers | |
|---|---|
| **Positive** | **Negative** |
| *MSC* | |
| CD73 | CD45 |
| CD90 | CD34 |
| CD105 | CD11b or CD14 |
| CD44 | CD19 or CD79 |
| CD146 | HLA-DR |
| CD29 | CD31 |
| HLA-ABC | |
| *Pluripotency* | |
| *Oct4* | Vimentin |
| *SOX2* | Collagen1 |
| Rex1 | N-cadherin |
| Nanog | |
| Lin28 | |
| CD24 | |
| SSEA3 and 4 | |
| Tra-1-60 or -81 | |
| E-cadherin | |

## 31.15 Critical parameters for future preclinical production of induced pluripotent stem cell–mesenchymal stem/stromal cells

Although pluripotent stem-cell-derived MSCs can be generated easily by a number of methods, deficiencies in the literature make it difficult to assess the efficiency of the differentiation. First, most papers do not compare cell surface markers with those of undifferentiated iPSCs or ESCs, nor with primary MSCs derived from adult tissues. It is important to determine that the cells are indeed differentiating from pluripotent stem cells to MSCs. We found that only CD73 changed expression. CD105 and CD90 are commonly expressed on different pluripotent stem cell lines [68]. Proposed minimal markers to assess mesenchymal, non-mesenchymal, and pluripotent markers are shown in Table 31.2.

Second, the ISCT criteria were often not used to confirm the presence of the four key MSC-defining parameters of plastic adherence, fibroblastic morphology, positivity for characteristic cell surface marker, and negativity for hematopoietic and endothelial markers. However, the ISCT criteria are based on adult bone-marrow-derived MSCs. It is now clear that MSCs from different organs, donors, donor ages, and different culture conditions may have different patterns of marker expression or differentiation potential (see Chapters 1 and 7). For example, we found that iPSC–MSCs and ESC–MSCs derived using the EB method had extremely limited adipogenic capacity, similar to that of human term-placenta-derived MSCs [49].

Third, limited evidence has been provided on pluripotent stem cell markers, *in vivo* tumorgenicity assays, or karyotype stability early and late in culture for pluripotent stem-cell-derived MSCs. We found that a number of lines of both ESC–MSCs and iPSC–MSCs, derived via both the EB and SB431542 method, developed karyotypic abnormalities in long-term culture (passage 3 and above). This is not due to the methods of production but to the inherent nature of pluripotent stem cells in *in vitro* culture conditions. Recently, this method has been used by our collaborators to derive canine iPSC–MSCs for potential uses in veterinary regenerative medicine applications [69]. This could provide critical *in vivo* safety information prior to the clinical use of the differentiated progeny of iPSC–MSCs.

## 31.16 Plasticity of lineage commitment: reprogramming, deprogramming and dedifferentiation

Cells undergoing fate changes do so either by incorporating molecular or functional changes [70]. Primitive MSCs can be

obtained by isolating MSCs from fetal tissue, reprogramming committed cells to pluripotent cells followed by partial differentiation, or partial reprogramming (or dedifferentiation) of a committed MSC to enhance the desired fetal MSC-like phenotype [71]. For example, indirect conversion of a cell type will change its fate to another cell type, bypassing dedifferentiation and reprogramming. During human development, stem cells are destined to differentiate to a committed cell type, such as cardiomyocytes, neurons, or fibroblasts, whereas during reprogramming, a laboratory-induced phenomenon, committed fibroblasts change their cell fate from a differentiated cell type to a pluripotent stem cell.

## 31.17 How to develop "young" mesenchymal stem/stromal cells: going backward to go forward?

The generation of a pluripotent cell type might seem to be the first option for deriving a renewable source of MSCs from an adult source and then differentiating them to a primitive cell type. However, iPSC generation usually involves the complete reprogramming of a fully differentiated cell (e.g., a fibroblast) using retroviral, transposomal, or episomal transfer systems. Now chemical factors alone have enabled the reprogramming of cells to a pluripotent phenotype. Treatment with valproic acid (VPA) allows not only pluripotency gene expression but also drives the cell toward pluripotency [72,73]. VPA treatment of c-kit$^+$ amniotic fluid cells resulted in a cell population that was pluripotent and gave rise to all germ layers upon differentiation [72,73], although it lacked the ability to form teratomas, regarded as the gold standard for pluripotency but favorable for clinical translation.

VPA has histone-deacetylase-inhibiting effects. Histone deacetylases are known to repress pluripotency genes like *Oct4* while inhibiting GSK3b by phosphorylation of Sox and activatation of Wnt signalling [72]. This method, however, involved a long culture period and is more time consuming than other methods for deriving MSCs from primitive stem cells.

## 31.18 Small-molecules inhibitors for generating "young" stem cells

In addition to the use of transgenic systems, cells have been reprogrammed to pluripotency by many small molecules or chemical inhibitors. These small molecules are safer than viral transduction methods as no genetic information is integrated into the cell genome. While transforming a cell type to pluripotency is termed reprogramming, the transformation of a more mature cell type to a less mature cell type is termed de-differentiation. Complete reprogramming has been achieved in MSCs by reprogramming MSCs to iPSCs [74,75]. However, partial reprogramming or de-differentiation of a more mature cell type, like a myocyte or fibroblast, to a less mature cell type, like an MSC, has been attempted in only a few cases with small molecules [76–78]. Chen *et al.* showed that the small molecule reversine reverted lineage-committed murine myoblasts to an MSC-like stage [61]. Further studies reported that short-term treatment of primary murine and human dermal fibroblasts with reversine allowed them to revert to a progenitor capable of differentiating into skeletal muscle both *in vitro* and *in vivo* [76]. Interestingly, these studies described how reversine initially increased cell size up to nine times, but, once removed, the cells reverted back to their original size, function, and growth kinetics. These changes were attributed to reversine being a mitogen-activated protein kinase 1 and non-muscle myosin II inhibitor.

Other small molecules have been reported to induce de-differentiation and change stem cell potency and self-renewal. (2′*Z*,3′*E*)-6-bromoindirubin-3′-oxime has been shown to be responsible for enhancing ESC self-renewal [79–81], a characteristic of primitive stem cells. Platelet-derived growth factor receptor inhibitor-IV increased pluripotency gene expression in human adult bone-marrow-derived MSCs [82]. The activities of these molecules are together suggestive of a de-differentiation process whereby (1) a cell type with higher potency is achieved, (2) cells self-renew more quickly, and (3) pluripotent stem cell marker expression is enhanced.

## 31.19 Primitive stem cells and mesenchymal stem/stromal cell generation by physical factors

Hypoxia has also been shown to improve the stemness of MSCs by increasing the fraction of rapidly self-renewing cells [83] or stemness markers in progenitor cells [84]. Three-dimensional culture, culture in ESC media, and selecting for c-kit$^+$ or CD34$^+$ cells have been reported to enhance or produce more pluripotent MSCs [72,73,83,85–87].

## 31.20 Conclusions

There is a major unmet need for an effective method of supplying a large source of MSCs for clinical use [36]. The identification of various MSC types from different organs and of different ages (fetal to adult) has raised the question as to the optimal cellular source of MSCs (and see Chapters 3–5 and 7).

Pluripotent stem-cell-derived-MSCs have been obtained through a number of methods. We have detailed our results for developing a novel clinically applicable method, using the TGF-β signaling pathway inhibitor (SB431542), to produce patient-specific MSCs from a source free from ethical concerns. The iPSC-derived MSCs showed standard MSC characteristics, adhering to the criteria outlined by the ISCT for the identification of MSCs. Their potential for scale-up with growth kinetics ideal

for practical utility and their lack of teratoma-forming ability *in vivo* support further therapeutic development of these cells. They readily undergo mesodermal differentiation, with a strong propensity for osteogenic differentiation. Further investigation of these cells at an appropriate passage number to avoid loss of primitiveness and osteogenicity could position these cells as an alternative MSC source for future tissue repair and tissue engineering applications.

## 31.21 Future directions

The fields of stem cell biology and tissue engineering are increasingly converging at the forefront of regenerative medicine. Investigation into the derivation of potential cell sources suitable for clinical use is currently offering more realistic approaches to therapy. The refinement of derivation methods, and potential manipulation of these, could derive more fetal-like MSCs and would ensure optimal osteogenic differentiative capacity and the fastest kinetics of proliferation. An off-the-shelf tissue matching therapy is a reality, with 98% of the Caucasian population being covered by approximately 200 homozygous donors. Further experimentation is required to determine the homing and engraftment characteristics of iPSC–MSCs. This exciting and novel area of research is beginning to be therapeutically promising in areas of regenerative medicine and tissue repair.

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## References

1 M. Crisan, S. Yap, L. Casteilla, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.
2 C. Campagnoli, I. A. Roberts, S. Kumar, *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001;**98**:2396–2402.
3 M. Krampera, S. Marconi, A. Pasini, *et al.* Induction of neural-like differentiation in human mesenchymal stem cells derived from bone marrow, fat, spleen and thymus. *Bone* 2007;**40**:382–390.
4 C. B. Portmann-Lanz, A. Schoeberlein, A. Huber, *et al.* Placental mesenchymal stem cells as potential autologous graft for pre- and perinatal neuroregeneration. *Am J Obstet Gynecol* 2006;**194**: 664–673.
5 Z. Y. Zhang, S. H. Teoh, J. H. Hui, *et al.* The potential of human fetal mesenchymal stem cells for off-the-shelf bone tissue engineering application. *Biomaterials* 2012;**33**:2656–2672.
6 A. K. Chen, S. Reuveny, S. K. Oh. Application of human mesenchymal and pluripotent stem cell microcarrier cultures in cellular therapy: achievements and future direction. *Biotechnol Adv* 2013;**31**:1032–1046.
7 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**:e47559.
8 G. Brooke, M. Cook, C. Blair, *et al.* Therapeutic applications of mesenchymal stromal cells. *Semin Cell Dev Biol* 2007;**18**: 846–858.
9 A. Trounson, R. G. Thakar, G. Lomax, D. Gibbons. Clinical trials for stem cell therapies. *BMC Med* 2011;**9**:52.
10 G. Siegel, T. Kluba, U. Hermanutz-Klein, *et al.* Phenotype, donor age and gender affect function of human bone marrow-derived mesenchymal stromal cells. *BMC Med* 2013;**11**:146.
11 M. F. Pittenger. Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.
12 R. A. Pelekanos, J. Li, M. Gongora, *et al.* Comprehensive transcriptome and immunophenotype analysis of renal and cardiac MSC-like populations supports strong congruence with bone marrow MSC despite maintenance of distinct identities. *Stem Cell Res* 2012;**8**:58–73.
13 L. da Silva Meirelles, P. C. Chagastelles, N. B. Nardi. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**:2204–2213.
14 S. Kern, H. Eichler, J. Stoeve, *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**:1294–1301.
15 E. Ragni, T. Montemurro, E. Montelatici, *et al.* Differential microRNA signature of human mesenchymal stem cells from different sources reveals an "environmental-niche memory" for bone marrow stem cells. *Exp Cell Res* 2013;**319**:1562–1574.
16 B. Prunet-Marcassus, B. Cousin, D. Caton, *et al.* From heterogeneity to plasticity in adipose tissues: site-specific differences. *Exp Cell Res* 2006;**312**:727–736.
17 P. V. Guillot, C. De Bari, F. Dell'Accio, *et al.* Comparative osteogenic transcription profiling of various fetal and adult mesenchymal stem cell sources. *Differentiation* 2008;**76**:946–957.
18 S. Barlow, G. Brooke, K. Chatterjee, *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stem cells. *Stem Cells Dev* 2008;**17**:1095–1107.
19 G. Brooke, H. Tong, J. P. Levesque, K. Atkinson. Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. *Stem Cells Dev* 2008;**17**:929–940.
20 S. M. Millard, N. M. Fisk. Mesenchymal stem cells for systemic therapy: shotgun approach or magic bullets? *Bioessays* 2013;**35**: 173–182.
21 C. K. Hee, M. A. Jonikas, S. B. Nicoll. Influence of three-dimensional scaffold on the expression of osteogenic differentiation markers by human dermal fibroblasts. *Biomaterials* 2006;**27**:875–884.
22 R. J. Deans, A. B. Moseley. Mesenchymal stem cells: biology and potential clinical uses. *Exp Hematol* 2000;**28**:875–884.
23 R. Quarto, M. Mastrogiacomo, R. Cancedda, *et al.* Repair of large bone defects with the use of autologous bone marrow stromal cells. *N Engl J Med* 2001;**344**:385–386.
24 Z. Y. Zhang, S. H. Teoh, M. S. Chong, *et al.* Neo-vascularization and bone formation mediated by fetal mesenchymal stem cell

tissue-engineered bone grafts in critical-size femoral defects. *Biomaterials* 2010;**31**:608–620.

25 P. Bianco, P. G. Robey, P. J. Simmons. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008;**2**:313–319.

26 P. A. Zuk, M. Zhu, H. Mizuno, *et al.* Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng* 2001;**7**:211–228.

27 A. Erices, P. Conget, J. J. Minguell. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;**109**:235–242.

28 S. A. Kuznetsov, M. H. Mankani, S. Gronthos, *et al.* Circulating skeletal stem cells. *J Cell Biol* 2001;**153**:1133–1140.

29 H. E. Young, T. A. Steele, R. A. Bray, *et al.* Human reserve pluripotent mesenchymal stem cells are present in the connective tissues of skeletal muscle and dermis derived from fetal, adult, and geriatric donors. *Anat Rec* 2001;**264**:51–62.

30 S. Gronthos, M. Mankani, J. Brahim, *et al.* Postnatal human dental pulp stem cells (DPSCs) *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2000;**97**:13625–13630.

31 P. V. Guillot, C. Gotherstrom, J. Chan, *et al.* Human first-trimester fetal MSC express pluripotency markers and grow faster and have longer telomeres than adult MSC. *Stem Cells* 2007;**25**:646–654.

32 A. D. Ho, W. Wagner, W. Franke. Heterogeneity of mesenchymal stromal cell preparations. *Cytotherapy* 2008;**10**:320–330.

33 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

34 E. M. Horwitz, K. Le Blanc, M. Dominici, *et al.* Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 2005;**7**:393–395.

35 E. Y. Waese, R. A. Kandel, W. L. Stanford. Application of stem cells in bone repair. *Skeletal Radiol* 2008;**37**:601–608.

36 Z. Y. Zhang, S. H. Teoh, M. S. Chong, *et al.* Superior osteogenic capacity for bone tissue engineering of fetal compared with perinatal and adult mesenchymal stem cells. *Stem Cells* 2009;**27**:126–137.

37 N. L. Boyd, K. R. Robbins, S. K. Dhara, *et al.* Human embryonic stem cell-derived mesoderm-like epithelium transitions to mesenchymal progenitor cells. *Tissue Eng Part A* 2009;**15**:1897–1907.

38 Q. Lian, E. Lye, K. Suan Yeo, *et al.* Derivation of clinically compliant MSCs from $CD105^+$, $CD24^-$ differentiated human ESCs. *Stem Cells* 2007;**25**:425–436.

39 T. Barberi, M. Bradbury, Z. Dincer, *et al.* Derivation of engraftable skeletal myoblasts from human embryonic stem cells. *Nat Med* 2007;**13**:642–648.

40 T. Barberi, L. M. Willis, N. D. Socci, L. Studer. Derivation of multipotent mesenchymal precursors from human embryonic stem cells. *PLoS Med* 2005;**2**:e161.

41 N. S. Hwang, S. Varghese, Z. Zhang, J. Elisseeff. Chondrogenic differentiation of human embryonic stem cell-derived cells in arginine–glycine–aspartate-modified hydrogels. *Tissue Eng* 2006;**12**:2695–2706.

42 E. N. Olivier, A. C. Rybicki, E. E. Bouhassira. Differentiation of human embryonic stem cells into bipotent mesenchymal stem cells. *Stem Cells* 2006;**24**:1914–1922.

43 C. Xu, J. Jiang, V. Sottile, *et al.* Immortalized fibroblast-like cells derived from human embryonic stem cells support undifferentiated cell growth. *Stem Cells* 2004;**22**:972–980.

44 N. S. Hwang, S. Varghese, H. J. Lee, *et al. In vivo* commitment and functional tissue regeneration using human embryonic stem cell-derived mesenchymal cells. *Proc Natl Acad Sci U S A* 2008;**105**:20641–20646.

45 S. E. Brown, W. Tong, P. H. Krebsbach. The derivation of mesenchymal stem cells from human embryonic stem cells. *Cells Tissues Organs* 2009;**189**:256–260.

46 P. Arpornmaeklong, S. E. Brown, Z. Wang, P. H. Krebsbach. Phenotypic characterization, osteoblastic differentiation, and bone regeneration capacity of human embryonic stem cell-derived mesenchymal stem cells. *Stem Cells Dev* 2009;**18**:955–968.

47 R. Kalluri, R. A. Weinberg. The basics of epithelial-mesenchymal transition. *J Clin Invest* 2009;**119**:1420–1428.

48 K. Takahashi, S. Yamanaka. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–676.

49 J. Yu, M. A. Vodyanik, K. Smuga-Otto, *et al.* Induced pluripotent stem cell lines derived from human somatic cells. *Science* 2007;**318**:1917–1920.

50 K. Takahashi, K. Tanabe, M. Ohnuki, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**:861–872.

51 K. Kaji, K. Norrby, A. Paca, *et al.* Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature* 2009;**458**:771–775.

52 T. Watabe, A. Nishihara, K. Mishima, *et al.* TGF-β receptor kinase inhibitor enhances growth and integrity of embryonic stem cell-derived endothelial cells. *J Cell Biol* 2003;**163**:1303–1311.

53 C. A. Harrison, P. C. Gray, W. W. Vale, D. M. Robertson. Antagonists of activin signaling: mechanisms and potential biological applications. *Trends Endocrinol Metab* 2005;**16**:73–78.

54 L. Vallier, T. Touboul, S. Brown, *et al.* Signaling pathways controlling pluripotency and early cell fate decisions of human induced pluripotent stem cells. *Stem Cells* 2009;**27**:2655–2666.

55 L. Vallier, M. Alexander, R. A. Pedersen. Activin/Nodal and FGF pathways cooperate to maintain pluripotency of human embryonic stem cells. *J Cell Sci* 2005;**118**:4495–4509.

56 E. Reissmann, H. Jörnvall, A. Blokzijl, *et al.* The orphan receptor ALK7 and the Activin receptor ALK4 mediate signaling by Nodal proteins during vertebrate development. *Genes Dev* 2001;**15**:2010–2022.

57 A. F. Schier. Nodal signaling in vertebrate development. *Annu Rev Cell Dev Biol* 2003;**19**:589–621.

58 L. Vallier, S. Mendjan, S. Brown, *et al.* Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. *Development* 2009;**136**:1339–1349.

59 R. H. Xu, T. L. Sampsell-Barron, F. Gu, *et al. NANOG* is a direct target of TGFβ/Activin-mediated SMAD signaling in human ESCs. *Cell Stem Cell* 2008;**3**:196–206.

60 A. Mahmood, L. Harkness, H. D. Schrøder, *et al.* Enhanced differentiation of human embryonic stem cells to mesenchymal progenitors by inhibition of TGF-β/activin/nodal signaling using SB-431542. *J Bone Miner Res* 2010;**25**:1216–1233.

61 Y. S. Chen, R. A. Pelekanos, R. L. Ellis, *et al.* Small molecule mesengenic induction of human induced pluripotent stem cells to generate mesenchymal stem/stromal cells. *Stem Cells Transl Med* 2012;**1**:83–95.

62 P. Samavarchi-Tehrani, A. Golipour, L. David, *et al*. Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. *Cell Stem Cell* 2010;**7**:64–77.

63 R. Li, J. Liang, S. Ni, *et al*. A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. *Cell Stem Cell* 2010;**7**:51–63.

64 X. Liu, Y. Yue, B. Li, *et al*. A G protein-coupled receptor is a plasma membrane receptor for the plant hormone abscisic acid. *Science* 2007;**315**:1712–1716.

65 K. Le Blanc. Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 2003;**5**:485–489.

66 C. Aguilar-Gallardo, M. Poo, E. Gomez, *et al*. Derivation, characterization, differentiation, and registration of seven human embryonic stem cell lines (VAL-3, -4, -5, -6 M, -7, -8, and -9) on human feeder. *In Vitro Cell Dev Biol Anim* 2010;**46**:317–326.

67 Y. Mayshar, U. Ben-David, N. Lavon, *et al*. Identification and classification of chromosomal aberrations in human induced pluripotent stem cells. *Cell Stem Cell* 2010;**7**:521–531.

68 J. Huang, L. Yuan, X. Wang, *et al*. Icaritin and its glycosides enhance osteoblastic, but suppress osteoclastic, differentiation and activity *in vitro*. *Life Sci* 2007;**81**:832–840.

69 D. J. Whitworth, J. E. Frith, T. J. Frith, *et al*. Derivation of mesenchymal stromal cells from canine induced pluripotent stem cells by inhibition of the TGFβ/activin signaling pathway. *Stem Cells Dev* 2014;**23**:3021–3033.

70 K. Hochedlinger, K. Plath. Epigenetic reprogramming and induced pluripotency. *Development* 2009;**136**:509–523.

71 J. Ladewig, P. Koch, O. Brustle. Leveling Waddington: the emergence of direct programming and the loss of cell fate hierarchies. *Nat Rev Mol Cell Biol* 2013;**14**:225–236.

72 D. Moschidou, S. Mukherjee, M. P. Blundell, *et al*. Valproic acid confers functional pluripotency to human amniotic fluid stem cells in a transgene-free approach. *Mol Ther* 2012;**20**:1953–1967.

73 D. Moschidou, S. Mukherjee, M. P. Blundell, *et al*. Human mid-trimester amniotic fluid stem cells cultured under embryonic stem cell conditions with valproic acid acquire pluripotent characteristics. *Stem Cells Dev* 2013;**22**:444–458.

74 T. Shofuda, D. Kanematsu, H. Fukusumi, *et al*. Human decidua-derived mesenchymal cells are a promising source for the generation and cell banking of human induced pluripotent stem cells. *Cell Medicine* 2013;**4**:125–147.

75 V. Spinelli, P. V. Guillot, P. De Coppi. Induced pluripotent stem (iPS) cells from human fetal stem cells (hFSCs). *Organogenesis* 2013;**9**:101–110.

76 L. Anastasia, M. Sampaolesi, N. Papini, *et al*. Reversine-treated fibroblasts acquire myogenic competence in vitro and in regenerating skeletal muscle. *Cell Death Differ* 2006;**13**:2042–2051.

77 M. Saraiya, R. Nasser, Y. Zeng, *et al*. Reversine enhances generation of progenitor-like cells by dedifferentiation of annulus fibrosus cells. *Tissue Eng Part A* 2010;**16**:1443–1455.

78 S. Chen, S. Takanashi, Q. Zhang, *et al*. Reversine increases the plasticity of lineage-committed mammalian cells. *Proc Natl Acad Sci U S A* 2007;**104**:10482–10487.

79 W. Goessling, T. E. North, S. Loewer, *et al*. Genetic interaction of PGE2 and Wnt signaling regulates developmental specification of stem cells and regeneration. *Cell* 2009;**136**:1136–1147.

80 N. Sato, L. Meijer, L. Skaltsounis, *et al*. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med* 2004;**10**:55–63.

81 U. Ullmann, C. Gilles, M. De Rycke, *et al*. GSK-3-specific inhibitor-supplemented hESC medium prevents the epithelial-mesenchymal transition process and the up-regulation of matrix metalloproteinases in hESCs cultured in feeder-free conditions. *Mol Hum Reprod* 2008;**14**:169–179.

82 S. G. Ball, A. Shuttleworth, C. M. Kielty. Inhibition of platelet-derived growth factor receptor signaling regulates *Oct4* and Nanog expression, cell shape, and mesenchymal stem cell potency. *Stem Cells* 2012;**30**:548–560.

83 M. M. Saller, W. C. Prall, D. Docheva, *et al*. Increased stemness and migration of human mesenchymal stem cells in hypoxia is associated with altered integrin expression. *Biochem Biophys Res Commun* 2012;**423**:379–385.

84 H. C. Chen, H. K. Sytwu, J. L. Chang, *et al*. Hypoxia enhances the stemness markers of cochlear stem/progenitor cells and expands sphere formation through activation of hypoxia-inducible factor-1α. *Hear Res* 2011;**275**:43–52.

85 I. Nazarov, J. W. Lee, E. Soupene, *et al*. Multipotent stromal stem cells from human placenta demonstrate high therapeutic potential. *Stem Cells Transl Med* 2012;**1**:359–372.

86 E. Resca, M. Zavatti, L. Bertoni, *et al*. Enrichment in c-Kit$^{+}$ enhances mesodermal and neural differentiation of human chorionic placental cells. *Placenta* 2013;**34**:526–535.

87 J. Patel, E. Seppanen, M. S. Chong, *et al*. Prospective surface marker-based isolation and expansion of fetal endothelial colony-forming cells from human term placenta. *Stem Cells Transl Med* 2013;**2**: 839–847.

CHAPTER 32

# The role of mesenchymal stem cells in hematopoiesis

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## 32.1 Introduction

Hematopoiesis, or the continual renewal of blood cells that takes place in the bone marrow after birth, must face two conflicting demands. The most obvious demand is the continuous and balanced replacement of blood leukocytes, erythrocytes, and platelets, since blood cells can have a rapid turnover from 45 days for erythrocytes to less than 24 h for platelets. As a consequence, approximately $2.5 \times 10^6$ erythrocytes, $0.5 \times 10^6$ granulocytes, and $15–20 \times 10^6$ platelets are produced every second in an average adult human. This equates to approximately 500 billion blood leukocytes, erythrocytes, and platelets produced per day in an average human adult to simply maintain healthy levels of these cells in the circulation. This astounding replacement rate is the product of a hierarchy of progressive differentiation and lineage commitment from the rare multipotent hematopoietic stem cells (HSCs), to the highly proliferative hematopoietic progenitor cells (HPCs). Owing to their multipotentiality and their high capacity to self-renew, HSCs are the only cells able to fully reconstitute the whole hematopoietic and immune systems life-long upon transplantation, whereas the more mature HPCs actively proliferate and differentiate to rapidly repopulate the blood and immune cells but are incapable of self-renewing in the long term and only maintain the hematopoietic system for a few weeks. Owing to the rarity of HSCs in the adult bone marrow (approximately 1 in 10 000–20 000 cells), their genomic and functional integrity must be preserved to maintain efficient life-long hematopoiesis and avoid premature hematopoietic exhaustion and bone marrow failure. The second demand that hematopoiesis must face is the preservation of these rare HSCs from cellular damage, oxidative stress, and increased rates of DNA replication errors associated with extensive proliferation.

In rodents and humans these two conflicting demands are resolved by the maintenance of a dormant pool of HSCs that divide rarely in steady-state adults with approximately one division every 145 days in mice [1] and every 40 weeks in humans [2]. A population of less dormant HSCs replicate more frequently to replace the pool of actively dividing multipotent progenitors (MPPs) [1]. Although MPPs generate the whole hierarchy of actively dividing lineage-restricted HPCs that subsequently regenerate the large number of blood cells consumed by the body, MPPs are not stem cells and, as such, have a very limited self-renewal capacity. Consequently, they cannot reconstitute the whole hematopoietic system beyond a few weeks after transplantation.

The maintenance of this rare pool of highly dormant HSCs has led to some seemingly counterintuitive observations. A recent

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technique involving tagging single clonal HSCs with a doxycycline-inducible hyperactive sleeping beauty (HSB) transposase enables the random integration of a DNA sequence tag into the HSC genome, thus allowing the tracking of sequential HSC clones and their progenies according to the integration sites of the tags. The advantage of this technique over conventional HSC barcoding methods is that it is performed on mice in steady-state hematopoiesis and does not require HSC isolation, retroviral barcoding, and transplantation after lethal irradiation of recipients, which forces barcoded HSCs to proliferate in order to reconstitute the bone marrow of myeloablated recipients.

This HSB-mediated HSC tagging in mice in steady state hematopoiesis has shown that granulocytes, which have a short half-life (10 h to 5–6 days depending on the method used [3]), are renewed by more proliferative progenitors, not the dormant HSCs over a long period of 45 weeks in steady state, an unexpected feature unique to granulocytes and not shared with lymphocytes or monocytes, which are renewed from common HSC clones [4]. While the coexistence of deeply dormant HSCs with more active HSCs is observed during steady-state hematopoiesis, this system is highly adaptable in response to stress, resulting in the activation of dormant HSCs to proliferate when rapid expansion is required, such as during early life when the body grows or to repair the hematopoietic system following myeloablation [1], infections [5,6], or HSC transplantation [4].

## 32.2 Hematopoietic stem cells need a niche

The dynamics of the hematopoietic system in steady state requires precise tuning to maintain the pool of deeply dormant HSCs, while allowing the more proliferative HSCs to actively regenerate hematopoietic tissue. Moreover, this system needs to be adaptable and sensitive to external demands, to reversibly activate dormant HSCs into a proliferative state in response to stress, and return them to a dormant state once the system has normalized. This exquisite balance of HSC quiescence versus proliferation, and self-renewal versus differentiation, is in large part regulated by specific HSC niches within the bone marrow. HSCs cannot be maintained for very long periods of time outside these specialized environments.

While *in vitro* cultures of HSCs with soluble recombinant cytokines generate a large number of proliferative HPCs and leukocytes at various maturational stages [7–10], human or mouse HSCs that maintain their self-renewal potential and their capacity to reconstitute the whole hematopoietic system upon transplantation are lost over extended periods of culture in the absence of stromal cells [11–16]. The rare occasions of successful maintenance or expansion of transplantable human or mouse HSCs have been performed in co-cultures with mesenchymal progenitor cells [17] or immortalized endothelial cells [18,19].

Earlier studies have shown that autologous transplantation of large pieces of bone marrow fragments into various extramedullary tissues in rats resulted in engraftment of the stromal tissue, followed by complete reconstitution of the hematopoietic bone marrow [20], demonstrating that bone marrow stromal elements are necessary to support HSC survival *in vivo*. This clearly demonstrated that the intricate cellular and physical interactions taking place *in situ* in the bone marrow between HSCs, HPCs, and the medullary stroma are required to maintain hematopoiesis *in vivo*. Subsequent studies have shown that niche cellular components, the extracellular matrix [21], and physicochemical cues such as oxygenation [22–25] critically regulate HSC dormancy, self-renewal, and reconstituting potential.

Although the nature and the components of HSC niches remained elusive for over two decades after their conceptualization by Schofield in 1978 [26], recent technical advances in the last decade have allowed further insights into this. In particular, (a) novel genetic tools in the mouse and (b) large panels of antibodies against human antigens together with potent human–mouse xenotransplantation systems have revealed the basic characteristics of HSC niches. It is increasingly clear that while mesenchymal stem cells (MSCs) in the bone marrow are pivotal components of HSC niches, they do not act alone to regulate HSCs in their niches but in concert with many other cell types. An increasing collection of other bone marrow stromal cells, such as endothelial cells, more mature mesenchymal progenitors, megakaryocytes, and Schwann cells, also play important roles in directly regulating HSCs, while neurons and macrophages are important regulators of the niche. This chapter will review some recent advances in dissecting the interactions between MSCs and HSCs firstly in mice and then in humans.

## 32.3 A mesenchymal hierarchy

In the literature the term "mesenchymal stem cell" has often been incorrectly used to refer to mesenchymal progenitor cells, defined by a set of phenotypic cell surface markers and the capacity to proliferate in culture and differentiate into cells of mesenchymal lineages, such as osteoblasts, chondrocytes, or adipocytes. However, these characteristics are not sufficient to define these cells as "stem cells." Multilineage differentiation from a population of cells may be due to the presence of a mixture of different lineage-restricted progenitors with limited self-renewal and reconstitution capacity *in vivo* rather than a single multipotent self-renewing stem cell. As a "stem cell," a single MSC must be able both self-renew into daughter stem cells and to generate a clone with the ability to differentiate into a variety of mesenchymal lineages and robustly reconstitute a mesenchymal tissue such as a bone or an ossicle once transplanted [27]. Initial characterization of mesenchymal progenitor cells included assays such the colony-forming unit–fibroblast (CFU-F) assay that defined nonhematopoietic fibroblast-shaped progenitor cells able to form colonies in clonal conditions, to be serially replatable, and to differentiate into different mesenchymal lineages (such as osteoblasts, chondrocytes, or adipocytes) when

cultured in appropriate conducive conditions [28,29]. The discovery of monoclonal antibodies such as STRO-1 and CD146 that could be used to enrich human bone marrow stromal cells for CFU-F and mesenchymal progenitors provided the first tool to further characterize mesenchymal progenitor cells [30,31]. It is, however, in the mouse that the clearest demonstration of clonal MSCs with multilineage mesenchymal differentiation potential and the ability to support the formation of an ossicle containing an active hematopoietic bone marrow was performed, as discussed herein.

## 32.4 Identification of mesenchymal stem cells and their relationship with hematopoietic stem cells in the mouse

In a seminal work, Paul Frenette's group found a rare population of bone marrow stromal cells expressing the intermediate filament protein nestin, which is abundantly expressed by neuroectoderm stem cells during central nervous system development [32,33]. These cells were multipotent, self-renewing, and capable of forming ossicles with functional hematopoietic marrow following subcutaneous transplantation [34]. Using a transgenic mouse strain in which green fluorescent protein (GFP) reporter expression was driven by a *Nestin* gene promoter (Nes-GFP mice), they noticed that the adult mouse bone marrow contained rare nestin$^+$ GFP$^+$ stromal cells that were negative for the pan-leukocyte marker CD45, as well as endothelial markers such as VE-cadherin (CD144) and platelet endothelium adhesion molecule-1 (CD31). These nestin$^+$ GFP$^+$ cells represented approximately 0.08% of bone marrow nucleated cells and were wrapped around endothelial sinusoids in the bone marrow, suggesting they may be pericytes. Using fluorescence-activated cell sorting (FACS), these cells were sorted from the bone marrow of Nes-GFP mice. Subsequent two-dimensional culture of GFP$^+$ nestin$^+$ cells on plastic wells showed these cells could not self-renew and rapidly differentiated into osteoblastic and chondrogenic lineage progenitors, resulting in a rapid loss of all GFP$^+$ nestin$^+$ cells. However, in three-dimensional (3D) cultures supplemented with appropriate growth factors and chicken embryo extracts, these GFP$^+$ nestin$^+$ cells could spontaneously assemble into spheres called mesenspheres and expand. These mesenspheres, clonally grown from single GFP$^+$ nestin$^+$ cells, contained a mixture of osteoblasts, chondrocytes, and adipocytes at different maturational stages as well as maintained a pool of undifferentiated GFP$^+$ nestin$^+$ cells. Remarkably, when these clonal mesenspheres were attached to a phosphocalcic implant and transplanted subcutaneously, they developed into ossicles containing osteoblastic and chondrocytic cells derived from the initial GFP$^+$ nestin$^+$ donor cells. Furthermore, nestin$^+$ GFP$^+$ cells from these mesensphere-derived ossicles were re-sorted and formed new mesenspheres that could be transplanted into secondary recipient mice and form ossicles with functional hematopoietic marrow [34]. Therefore, under the most stringent clonal conditions, a proportion of nestin$^+$ GFP$^+$ cells in the bone marrow of adult Nes-GFP mice are capable of self-renewal and trilineage differentiation *in vitro* and *in vivo*, thus fulfilling the functional characteristic of true MSCs [27].

Intriguingly, despite the rarity of nestin$^+$ cells and even rarer HSCs rigorously defined as lineage-negative, 60% of HSCs were within five cell diameters of a nestin$^+$ cell, suggesting a close physical proximity between these two stem cell populations in mouse bone marrow.

(Lineage negativity is defined as absence of the following lineage markers: CD3 (a pan T cell marker), CD45R (the common leukocyte antigen), CD11b (integrin $\alpha_M$ on monocytes, granulocytes, macrophages, and natural killer cells), Ly6C/G (granulocyte receptor Gr-1 expressed on most myeloid cells in the bone marrow and all peripheral granulocytes), CD41 (integrin $\alpha_{IIb}$ or platelet GPIIb on megakaryocytes), Ter119 (erythroid cells), CD48 (B lymphocyte activation marker also known as BLAST-1 and signaling lymphocyte activation molecule 2, SLAMF2), and CD150 (signaling lymphocyte activation molecule 1, SLAMF1) [35].)

Importantly, these nestin$^+$ MSCs had the highest expression levels of chemokines (such as CXCL12), growth factors (such as kit ligand and angiopoietin-1), cell adhesion molecules (such as vascular cell adhesion molecule-1 – VCAM-1; CD106), and connexin 43 [34] necessary to maintain HSCs within their bone marrow niches [36]. Taken together, these findings suggest that these nestin$^+$ MSCs are pivotal to maintain viable HSCs in their niche. However, definitive proof that these nestin$^+$ MSCs are essential to HSC maintenance was provided by selectively ablating nestin$^+$ MSCs *in vivo*. As germinal ablation of nestin$^+$ cells is embryo-lethal due to its expression in the embryonic neuroectoderm, MSCs were selectively depleted in adult mice using diphtheria toxin. As mice do not express receptors for diphtheria toxin and are consequently resistant to this very potent toxin [37], a classic strategy of inducing expression of simian diphtheria toxin receptor (DTR) specifically in nestin$^+$ cells was developed in which DTR$^+$ cells could then be selectively ablated *in vivo* by administration of diphtheria toxin [34]. Generation of these mice involved crossing two different strains of mutant mice. The first strain expressed a fusion chimera between Cre recombinase and a mutant estrogen receptor that binds the estrogen antagonist tamoxifen but not endogenous estrogen (Cre$^{ERT2}$). In order to restrict its expression to nestin$^+$ cells, this chimeric construct was placed under the control of the *Nestin* gene enhancer (*Nes-Cre$^{ERT2}$* mice). These mice were crossed with a strain containing a Cre-inducible simian DTR (iDTR) (Figure 32.1). In the resulting *Nes-Cre$^{ERT2}$ iDTR* mice, the Cre$^{ERT2}$ fusion protein is specifically expressed in nestin$^+$ cells but trapped in the cytoplasm in the absence of tamoxifen. However when tamoxifen is administered to the mice it binds to Cre$^{ERT2}$, resulting in its translocation to the nucleus where it can recombine DNA between two loxP sequences. As the simian DTR transgene is preceded by a few STOP codons flanked by two

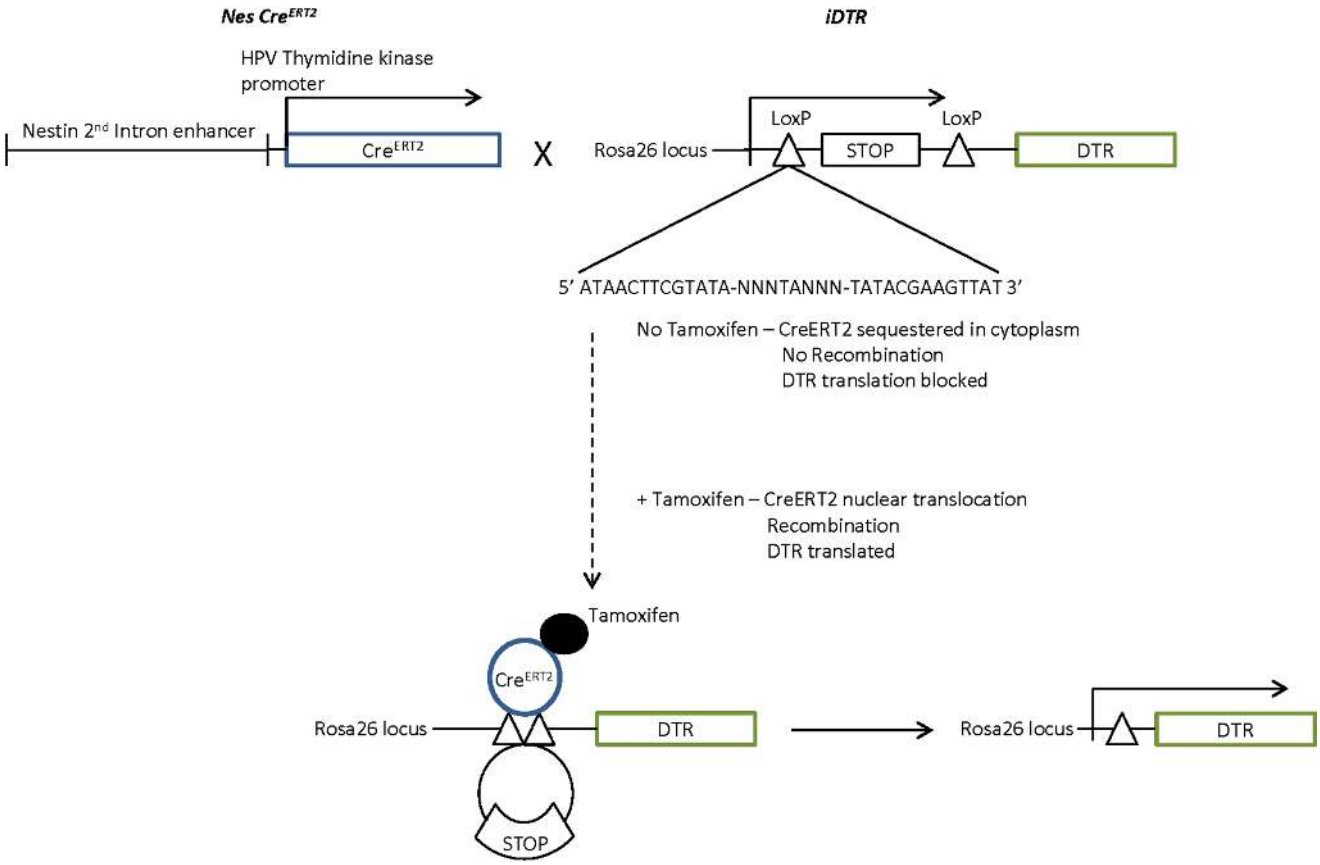



**Figure 32.1** Example of use of the Cre–Lox system to study the role of MSCs and other niche cells in regulating HSCs. In this particular example taken from reference [34], mice carrying the *Nes-CreER*$^{T2}$ transgene are crossed with iDTR mice containing a tamoxifen-inducible simian diphtheria toxin receptor cDNA downstream of a STOP codon flanked by two LoxP sites (floxed) inserted in the Rosa-26 gene trap locus. An example of loxP sequence with typical palindromes at both ends is shown. In the absence of tamoxifen, the CreER$^{T2}$ protein remains sequestered in the cytoplasm and cannot recombine genomic DNA in the cell nucleus. Consequently, the mRNA transcribed from the Rosa-26 gene trap locus in iDTR mice cannot be translated into DTR protein. Cells are resistant to diphtheria toxin. When mice are treated with tamoxifen, the drug crosses plasma membranes, binds to CreER$^{T2}$ protein, and induces its translocation to the nucleus via the estrogen-binding domain of the CreER$^{T2}$ protein. Once in the nucleus, CreER$^{T2}$ recombines DNA between loxP sequences and removes the STOP codon upstream of the DTR cDNA. As a consequence, the mRNA transcribed from the Rosa-26 is translated into DTR protein and cells become sensitive to diphtheria-toxin-mediated killing. By choosing Cre mice in which Cre is expressed in a specific cell type, in a constitutive or inducible manner (see examples in Tables 32.1 and 32.2), it is possible to induce gene expression (e.g., fluorescent reporter genes to track cells or toxins or toxin receptors to ablate cells) or to delete a particular gene in a cell-specific manner.

loxP sequences, translation of the DTIR protein is blocked in iDTR mice.

However, administration of tamoxifen activates Cre$^{ERT2}$ exclusively in nestin$^{+}$ cells (as they are the only cells to express Cre$^{ERT2}$ in *Nes-Cre$^{ERT2}$* mice), unlocking expression of DTR and rendering nestin$^{+}$ cells sensitive to diphtheria toxin (see Figure 32.1). Using this classic genetic approach, Mendez-Ferrer *et al.* were able to specifically deplete nestin$^{+}$ MSCs *in vivo* in adult *Nes-Cre$^{ERT2}$ iDTR* mice by co-administration of tamoxifen and diphtheria toxin. Two weeks following diphtheria-toxin-mediated nestin$^{+}$ cell ablation, the number of phenotypic Lin$^{-}$ Sca1$^{+}$ Kit$^{+}$ CD48$^{-}$ CD50$^{+}$ HSCs was reduced by 50% in the bone marrow, and hematopoietic activity in the bone marrow, as measured by long-term culture initiating cell assay, was reduced by 75%. Furthermore, homing of wild-type HSCs in recipients with nestin$^{+}$ cell depletion was reduced by 90% [34]. Taken together, these experiments suggest that rare nestin$^{+}$ cells in the adult mouse bone marrow are (1) bona fide MSCs capable of self-renewal and mesenchymal reconstitution *in vivo*, (2) physically associated with rare HSCs, and (3) supportive of HSC homing and maintenance in the bone marrow.

## 32.5 More than one nestin$^{+}$ cell type and hematopoietic stem cell niche exist in the mouse bone marrow

More refined flow cytometry and 3D confocal fluorescent microscopy analyses of *Nes-GFP* transgenic mice revealed GFP positivity did not define a single population of bone marrow MSCs. The first population identified exhibited very high expression of nestin and GFP and consisted of much scarcer elongated

cells (called $Nes^{peri}$) wrapped around arterioles. A second population with moderate expression of nestin and GFP were more reticular and found associated with sinusoids (called $Nes^{retic}$) [38]. Despite the varying locations, shapes, and GFP expression, both sorted populations contained CFU-F activity and expressed HSC-supportive genes such as *CXCL12*, *KITL*, *ANGPT1* or *VCAM1*. However, the $Nes^{peri}$ cells had higher levels of CFU-F activity as well as CXCL12, KITL, ANGPT1, or VCAM1 mRNA expression than $Nes^{retic}$ cells. This suggested that different types of $GFP^+$ $nestin^+$ MSCs reside in two different perivascular locations, either peri-arteriolar or peri-sinusoidal. As it had been inferred by several studies that dormant HSCs and more active HSCs reside in distinct niches according to their position relative to vascular beds, blood perfusion, and oxygenation of the surrounding milieu [1,22,25,39–43], the possibility that arteriolar and sinusoidal niches could segregate dormant from active HSCs was further explored. Dormant HSCs can be detected by oral administration of 5-bromo-2′-deoxyuridine (BrdU) or 5-ethynyl-2′-deoxyuridine (EdU), two thymidine analogs that incorporate into the DNA of dividing cells and can be easily detected with specific monoclonal antibodies. When mice drink water containing BrdU or EdU over a 2-week period, most HSCs (dormant or active) are labelled with these nucleotide analogs *in vivo* as their slight cytotoxicity forces HSCs to divide [1,44]. However, after a long chase over 70 days with plain drinking water, dividing cells will rapidly lose BrdU or EdU staining in the genomic DNA whereas dormant cells will retain these two nucleotide analogs during this long chase period, enabling their identification by flow cytometry or immunohistofluorescence [1,25]. Using this approach in *Nes-GFP* mice, 60% of dormant HSCs were found to reside within 20 μm of $Nes^{peri}$ cells in arterioles. As quiescent HSCs are not necessarily dormant (dormancy implies quiescence over several months), the proportion of quiescent HSCs defined by the absence of expression of the cell cycling nuclear marker Ki67 was also measured. Similar to dormant HSCs, 40% of quiescent HSCs were within 20 μm of $Nes^{peri}$ cells in these arteriolar niches. Conversely, cycling $Ki67^+$ HSCs were more distant from $Nes^{peri}$ [38].

It was also noted that peri-arteriolar $Nes^{peri}$ MSCs expressed the pericyte marker NG2 but not the leptin receptor (LEPR), which was previously found to be expressed by perivascular cells presenting Kit ligand to HSCs [45]. Conversely, peri-sinusoidal $Nes^{retic}$ MSCs were negative for NG2 and positive for LEPR [38]. Finally, in mice expressing both *iDTR* (Cre-inducible DTR) and *NG2-CreERT2* transgene (tamoxifen-inducible Cre recombinase fusion protein under the promoter of the gene encoding the pericytes marker NG2), diphtheria toxin treatment depleted approximately 50% of $Nes^{peri}$ MSCs in the mouse bone marrow, decreased the frequency of HSCs within 20 μm of an arteriole, and decreased the frequency of quiescent $Ki67^-$ HSCs [38]. From these findings it was concluded that dormant HSCs reside preferentially in peri-arteriolar niches close to $Nes^{peri}$ MSCs. It must be noted, however, that the frequency of dormant or quiescent HSCs in the proximity of peri-sinusoidal $Nes^{retic}$ MSCs was not shown. Therefore, while EdU-label-retaining dormant and $Ki67^-$ quiescent HSCs may be enriched in peri-arteriolar niches compared with the rest of the bone marrow space, their frequency in peri-sinusoidal niches close to $Nes^{retic}$ MSCs was not measured. Therefore, whether peri-sinusoidal niches preferentially harbor active or proliferative HSCs remains unclear.

Nevertheless, as nestin is an intracellular intermediate filament protein, the use of specific anti-nestin antibody to prospectively isolate viable MSCs by FACS from human or genetically unmodified mice is not possible. Screening of previously known cell surface markers of mesenchymal progenitor cells identified platelet-derived growth factor receptor-α (PDGFRα or CD140a) and integrin $\alpha_V$ (CD51) as the most abundantly expressed markers on $nestin^+$ $GFP^+$ bone marrow MSCs. Sixty percent of $nestin^+$ cells are double positive for both PDGFRα and CD51. Reciprocally, 75% of $CD45^-$ $Ter119^-$ $CD31^-$ $PDGR\alpha^+$ $CD51^+$ cells are $nestin^+$ and express the highest levels of CXCL12, VCAM1, ANGPT1, and SCF mRNA [46], factors all supportive of HSCs. Furthermore, sorted bone marrow $CD45^-$ $Ter119^-$ $CD31^-$ $PDGFR\alpha^+$ $CD51^+$ cells recapitulated all the properties of sorted $nestin^+$ $GFP^+$ MSCs, such as the formation of clonal mesenspheres, self-renewal *in vitro* and *in vivo*, and formation of ossicles containing a hematopoietic bone marrow when transplanted subcutaneously [46]. Therefore, double positivity for PDGFRα and CD51 within the $CD45^-$ $Ter119^-$ $CD31^-$ nonendothelial and nonhematopoietic gate is a good surrogate of nestin expression in adult bone marrow cells and is, to date, the best phenotypic profile to prospectively isolate mouse MSCs from mice that do not carry the *Nes-GFP* transgene. Of note, Pinho *et al.* found that the Sca-1 antigen was not present in these $nestin^+$ MSCs. Therefore, it should be possible to isolate MSCs from any mouse strain, especially from those that do not express Sca-1, such as BALB/c mice.

## 32.6 Controversies surrounding $nestin^+$ mesenchymal stem cells and other genetic models for alternative mesenchymal stem cells

The characterization of $nestin^+$ MSCs in the mouse bone marrow and its role in hematopoiesis has, however, been challenged by a recent report from Sean Morrison's group. As the tyrosine kinase receptor c-Kit (CD117) expressed by all hematopoietic stem and progenitor cells (HSPCs) and the Kit ligand (also called stem cell factor) expressed in the bone marrow stroma are both critical to HSC survival, self-renewal, and proliferation [47–49], *Nes-Cre* mice were crossed with mice carrying floxed *Kitl* alleles (flanked by two loxP sites, enabling conditional gene excision with Cre recombinase) to specifically delete the *Kitl* gene in $nestin^+$ cells [45]. This did not cause a reduction of HSC frequency in the bone marrow, but instead reduced their frequency in the spleen [45]. This result is at odds with previous findings showing that HSC frequency in the bone marrow was reduced

following acute depletion of nestin$^+$ MSCs in adult mice [34]. A possible explanation for this discrepancy is that in the *Nes-Cre$^{ERT2}$ iDTR* mouse model, ablation of nestin$^+$ MSCs was not constitutive but induced after administration of tamoxifen and diphtheria toxin in adult mice, whereas in Morrison's model the *Kitl* deletion in nestin$^+$ cells was constitutive throughout embryonic development. Indeed, it has recently been shown that, in the developing limb buds in the mouse embryo, a proportion of nestin$^+$ cells are endothelial cells expressing CD31. Similarly, at day 3 after birth, many nestin$^+$ cells gave rise to CD31 endothelial cells that persisted for up to a month, whereas at this stage their contribution to osteoblasts and chondrocytes was relatively minor, with no contribution to adipocytes [50]. Therefore, the cell specificity of nestin expression varies with developmental stages as nestin specifically labels MSCs only in the adult bone marrow [51], not in the embryo or perinatally [50]. Thus, conditional deletion or ablation in the adult mouse to study effects in adult bone marrow MSCs should be performed with tamoxifen-inducible *Nes-CreERT2* mice rather than *Nes-Cre* mice.

A second controversy is the observation by Morrison's group that HSC frequency in the bone marrow was decreased in mice in which *Kitl* was deleted by LEPR$^+$ cells (*Lepr-Cre* mice) [45], thus suggesting that LEPR$^+$ mesenchymal cells are critical to HSC development and survival, whereas Frenette's group could not detect LEPR in peri-arteriolar Nes$^{peri}$ MSCs. Instead, this group detected LEPR in peri-sinusoidal Nes$^{retic}$ MSCs [45]. However, a recent study shows that LEPR is also expressed in different bone marrow stromal subsets during development [51] and that osteoblasts in the embryo and adults emerge from distinct waves of mesenchymal differentiation from distinct MSCs depending on the developmental stage [50,51]. Therefore, as with nestin, LEPR is expressed in different cell types depending on developmental stage, underpinning the need for inducible gene deletions in adults rather than constitutive deletions during early embryonic development. Consequently, the precise role of nestin$^+$ versus LEPR$^+$ MSCs in the adult bone marrow, and the extent to which they overlap or segregate to arteriolar versus sinusoidal niches and dormant versus proliferative HSC niches, needs further clarification.

Besides models targeting nestin and LEPR, other genetic tools have become available to test MSC function in the mouse bone marrow (Tables 32.1 and 32.2).

One such model targets the transcription factor Prrx1 that is expressed by the limb bud mesooderm in the developing embryo and is critical to skeletal development [52,53]. Crossing these *Prrx1-Cre* mice with a Cre inducible fluorescent reporter showed that most mesenchymal cells expressing CXCL12 in the bone marrow are derived from Prrx1$^+$ MSCs. Furthermore, conditional deletion of the *CXCL12* gene in Prrx1$^+$ mesenchymal cells led to a dramatic reduction in the frequency of HSCs, as well as common lymphoid progenitors and B cell progenitors in the bone marrow and increased cycling of the remaining HSCs [54]. *Prrx1-Cre* mice are therefore a useful tool to test the role of the mesenchymal lineage in regulating hematopoiesis. However, they do not enable the different contributions of the various differentiation stages of MSCs to be distinguished for their respective contribution.

**Table 32.1** Cre mouse strains used to study HSC niche cells.

| Strain | Induction system | Cell specificity |
|---|---|---|
| *Mx1-Cre* | pI:pC inducible | All cells |
| *Nes-Cre$^{ERT2}$* | Tamoxifen inducible | Nestin$^+$ cells |
| *Nes-Cre* | Constitutive | Nestin$^+$ cells |
| *NG2-Cre* | Constitutive | Pericytes |
| *LEPR-Cre* | Constitutive | Mesenchymal LEPR$^+$ cells |
| *Prrx1-Cre* | Constitutive | MSCs |
| *Osx-Cre* | Constitutive | Immature osteoprogenitors |
| *Col2.3-Cre* | Constitutive | Maturing osteoprogenitors |
| *Osteocalcin-Cre* | Constitutive | Mature osteoblasts |
| *Tie-2-Cre* | Constitutive | Endothelial and hematopoietic cells |
| *VE-cadh-Cre$^{ERT2}$* | Tamoxifen inducible | Endothelial cells |
| *VE-cadh-Cre* | Constitutive | Endothelial cells |
| *Cxcl4-Cre* | Constitutive | Megakaryocytes |
| *Th-Cre* | Constitutive | Dopaminergic, adrenergic nerves |

## 32.7 Other stromal cells regulate hematopoietic stem cells and additional tools to study their role in regulating hematopoiesis

The last decade of research has shown that in both mice and humans an increasing variety of cell types regulate HSCs in the

**Table 32.2** Genes targeted by Cre mouse strains to study HSC niches.

| Gene | |
|---|---|
| *iDTR* | Inducible diphtheria toxin receptor |
| *iGFP* | Inducible GFP |
| *iYFP* | Inducible yellow fluorescent protein |
| *iRFP* | Inducible red fluorescent protein |
| *KITL* | Kit-ligand |
| *JAG1* | Jagged-1 |
| *CXCL12* | Stromal derived factor 1 (SDF-1) |

bone marrow. With the increasing availability of genetically modified mouse strains expressing the Cre recombinase under the control of cell-specific gene promoters (Tables 32.1 and 32.2) the precise function of some of these cell types has been revealed.

### 32.7.1 Osteoblastic lineage and osteoblasts

Cells of the osteoblastic lineage and osteoblasts have long been suspected to play an important role in regulating hematopoiesis. This notion arose from observations by Brian Lord four decades ago that short-term reconstituting HPCs detected in the colony-forming unit–spleen assay were more abundant close to the endosteum than in the central bone marrow [55]. Subsequent studies showed that $Lin^-$ $Sca1^+$ $Kit^+$ cells, which not only encompass HSCs but also a large proportion of MPPs and lineage-restricted HPCs, homed preferentially within 100 μm of the endosteum in lethally irradiated and nonmyeloablated recipients [56,57]. Conditional overexpression of a constitutively active parathyroid hormone receptor (PTHR1) or inactivation of the bone morphogenetic protein receptor 1A in maturing osteoblasts in which the 2.3 kb fragment of the collagen IA gene promoter is active (*Col2.3*-Cre mice) led to increased frequency of $Lin^-$ $Sca1^+$ $Kit^+$ HPCs and HSCs in the bone marrow [58,59]. Conversely, *in vivo* depletion of maturing osteoblasts using the *Col2.3* promoter led to the progressive relocation of hematopoiesis from the bone marrow to the spleen [60] and an arrest of medullary B lymphopoiesis [61]. However, with publications refining the phenotype identifying HSCs, MPPs, and lineage-restricted HPCs more precisely and the availability of additional Cre transgenic strains, the role of mature osteoblasts in directly regulating HSCs has become more controversial. Mature osteoblasts are not the main source of Kit ligand and CXCL12 in the bone marrow, as they are both abundantly expressed by more immature mesenchymal cells such as MSCs [38], as well as pericytes and CXCL12-abundant reticular cells [62]. Furthermore, conditional deletion of the *KITL* or *CXCL12* genes in maturing or mature osteoblasts using *Col2.3-Cre* mice or *osteocalcin-Cre* mice did not alter the frequency of HSCs in the bone marrow [54,63], suggesting that other cellular sources of Kit ligand and CXCL12 (such as from MSCs and endothelial cells) are more important to support medullary HSCs. However, it must be noted that maturing osteoblasts play an important role in maintaining medullary lymphopoiesis. Conditional deletion of the *CXCL12* gene in maturing osteoblasts (using *Col2.3-Cre* mice) led to a loss of medullary lymphopoiesis [63], an effect also observed following *in vivo* genetic ablation or granulocyte colony-stimulating factor (G-CSF)-mediated ablation of osteoblasts [60,61,64]. It is also possible that more primitive osteoprogenitors expressing the osterix/Sp7 transcription factor play an important role in regulating the proliferation of MPPs and HSCs. While deletion of *CXCL12* gene in $osterix^+$ osteoprogenitors did not alter HSC frequency or cycling in the bone marrow, it did increase their exit from quiescence and entry into the $G_1$ phase of the cell cycle and their mobilization into the blood and spleen [54]. Furthermore, blockage of microRNA synthesis in osteoprogenitors by conditionally deleting the *DICER1* gene in $osterix^+$ osteoprogenitors led to severe hematopoietic insufficiency and myelodysplasia evolving to secondary acute myeloid leukemia. However, conditional deletion of the *DICER1* gene in mature osteoblasts expressing osteocalcin did not lead to hematopoietic abnormalities [65]. Likewise, deletion of the *Sbds* gene, involved in ribosome regulation, in $osterix^+$ osteoprogenitors also leads to severe hematopoietic insufficiency and myelodysplasia evolving to secondary acute myeloid leukemia [65]. Therefore, dysregulation of osteoprogenitors can cause hematopoietic deficiencies or abnormal HSC trafficking and mobilization into the blood.

### 32.7.2 Endothelial cells

Visualization of rare HSCs (using the $Lin^-$ $CD48^-$ $CD150^+$ phenotype) by confocal immunofluorescence microscopy in sections of adult mouse long bones or calvaria or whole mount adult marrows *in situ* revealed that a large proportion of these rare HSCs are located adjacent to a blood vessel often reported as a sinusoid [35,62]. This raised the question as to whether this perivascular location of HSCs is due to the perivascular location of MSCs that support HSCs or whether endothelial cells are also directly supporting HSCs. There is strong evidence that both human [18] and mouse [19] endothelial cells can support expansion of human and mouse HSCs in co-cultures. Furthermore, many chemokines, cell adhesion molecules, and growth factors critical to the retention of HSCs within the bone marrow tissue, such as CXCL12 [54,63,66,67], VCAM-1 [68,69], and kit ligand [45], were not only expressed by perivascular mesenchymal cells but also by some endothelial cells in the bone marrow (Figure 32.2).

Furthermore, E-selectin, an endothelial cell-specific adhesion molecule expressed by a subset of bone marrow endothelial cells, promotes HSC proliferation in the bone marrow *in vivo* as deletion of the E-selectin gene *SELE*, or administration of selective antagonists of E-selectin, increases HSC quiescence and resistance to cytotoxic drugs and irradiation *in vivo* [40]. Taken together, this strongly suggests that endothelial cells, independent of MSCs, are important regulators of HSCs and hematopoiesis. Proof of a direct role of endothelial cells in regulating HSCs was recently provided by crossing transgenic mice with endothelial-cell-specific expression of Cre recombinase or tamoxifen-inducible $Cre^{ERT2}$ with mice in which either the *KITL* or *JAG1* (encoding the Notch 1 ligand and Jagged 1) genes were floxed (Tables 32.1 and 32.2). Constitutive excision of the *KITL* gene in endothelial cells reduced the frequency of HSCs in the bone marrow and spleen, and this reduction worsened with age [45]. Therefore, ongoing endothelial expression of Kit ligand is required for full maintenance of HSCs. Likewise, constitutive deletion of the *JAG1* gene in endothelial cells reduced HSC frequency in the bone marrow, self-renewal potential upon serial transplantation, and impaired recovery and survival from sublethal irradiation or cytotoxic injury with 5-fluorouracyl [70]. Taken together, these models demonstrate that, concomitantly to

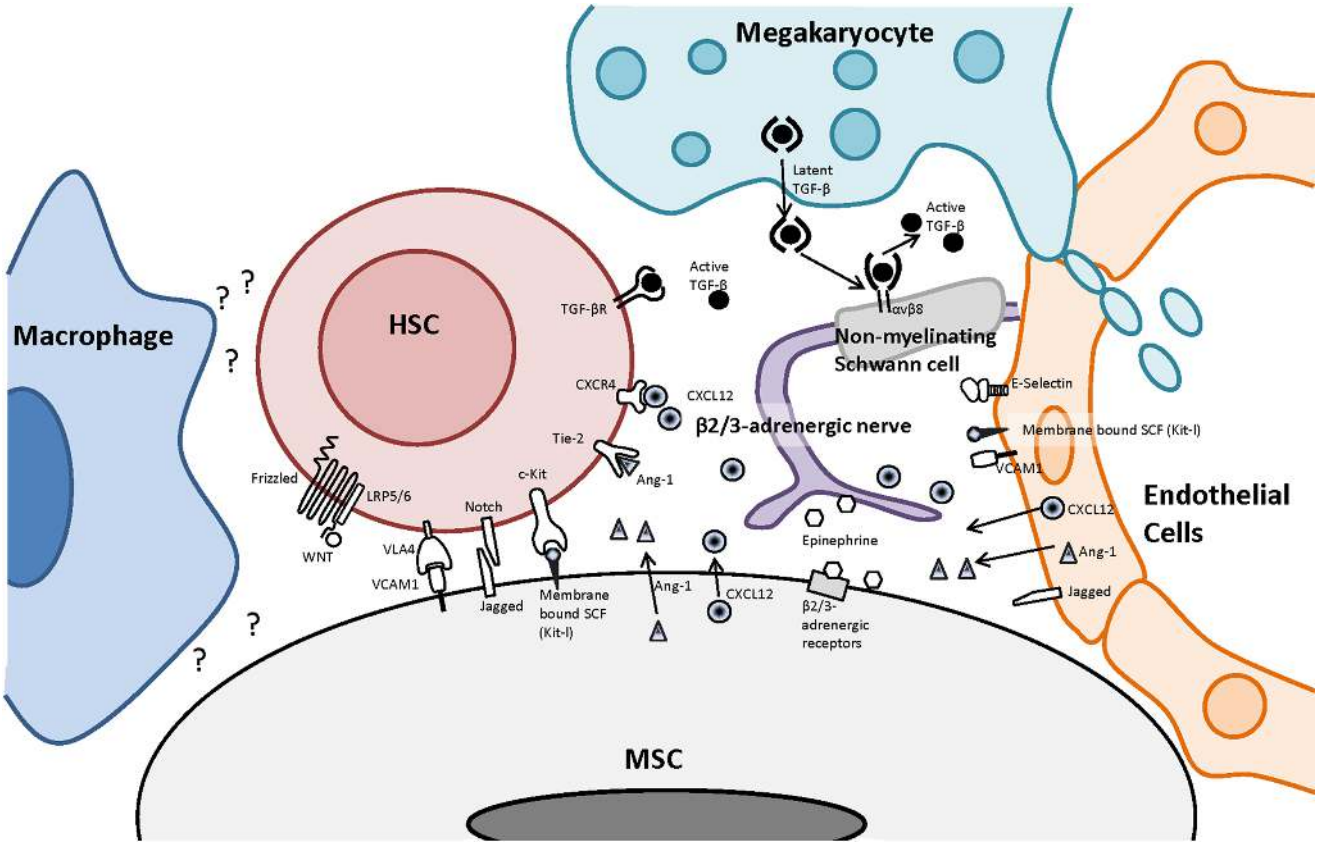



**Figure 32.2** Schematic view of HSC niches. MSCs expressing nestin play a central role in the HSC niche. MSCs are perivascular and can be present in bone marrow arterioles (arteriolar niches with nestin$^{bright}$ MSCs) or sinusoids (sinusoidal niches with nestin$^{dim}$ MSCs). MSCs, together with bone marrow endothelial cells, express key regulators of HSCs, such as kit ligand, angiopoietin-1 (Ang-1), CXCL12, or Jagged-1, as well as VCAM-1, which anchors HSCs within their niches. CXCL12 expression by MSCs and MSC quiescence are regulated by norepinephrine and epinephrine released from β2- and β3-adrenergic nerves. Specific macrophages also regulate MSCs and the niche via unknown mechanisms. E-selectin specifically expressed by bone marrow endothelial cells promotes HSC proliferation. Latent transforming growth factor-β (TGF-β) released by megakaryocytes binds to $\alpha_v\beta_8$ integrin on Schwann cells, enabling its activation. Active TGF-β maintains HSCs in a quiescent state.

MSCs, subsets of bone marrow endothelial cells independently support HSCs and hematopoiesis in the bone marrow.

### 32.7.3 Megakaryocytes, Schwann cells, and the transforming growth factor-β connection

It has been known for two decades that the three isoforms of TGF-β, particularly TGF-β1, are critical regulators of HSC quiescence and self-renewal potential by blocking HSC entry into the cell cycle [71–73] via the SMAD signaling pathway [74,75]. Following biosynthesis, TGF-β is secreted in a dimeric proform called small latent complex, which contains a latency associated peptide (LAP). This small latent complex is further bound to a latent TGF-β binding protein to form a large latent complex. Active dimeric TGF-β is released from a small or large latent complex by proteolysis or acidification. Megakaryocytes in the bone marrow are perivascular in order to shed platelets into the circulation [76] and are an important source of TGF-β [77]. Fifteen hours following HSC transplantation in nonablated recipients, 80% of HSCs are lodged within two cell diameters of a megakaryocyte [78], whereas in the naive bone marrow 20–25% of HSCs are within 5 μm or directly in contact with a megakaryocyte [79,80]. This close proximity is not fortuitous, as specific ablation of megakaryocytes resulted in HSC expansion in the bone marrow. Indeed, in two recent independent studies, *Cxcl4-Cre* (also called *Pf4-Cre*) transgenic mice with Cre-recombinase driven by the promoter of the *CXCL4* gene (also called platelet factor 4, specific for megakaryocytes) were crossed with *iDTR* mice (Tables 32.1 and 32.2). In these mice, administration of diphtheria toxin ablated megakaryocytes, resulting in thrombocytopenia and a 10-fold increase in phenotypic HSCs due to their exit from quiescence, and subsequent proliferation [79,80]. Furthermore increased proliferation of phenotypic HSCs was not associated with decreased self-renewal but rather with increased long-term reconstitution potential upon transplantation. Therefore megakaryocytes play an important role in maintaining HSC quiescence in the bone marrow. This effect was mediated by both TGF-β1 and CXCL4. Injection of active recombinant TGF-β1 could restore HSC quiescence and decrease HSC numbers to normal levels in megakaryocyte-ablated *Cxcl4-Cre iDTR* mice [80]. Similarly

deletion of the *CXCL4* gene in *Cxcl4*$^{-/-}$ mice recapitulated the increase in HSC proliferation and number observed in the bone marrow of megakaryocyte-ablated *Cxcl4-Cre iDTR* mice [79]. Whether TGF-β is produced by megakaryocytes in its active or latent form was not determined in these two studies [79,80]; however, antibodies specific for active TGF-β or for LAP stain distinct cell types in the mouse bone marrow. In particular, antibodies specific for active TGF-β do not stain megakaryocytes but exclusively long elongated nonmyelinated Schwann cells expressing glial fibrillary acidic protein which ensheath nerves around blood vessels in the bone marrow [81]. Furthermore, these Schawnn cells express integrin $\alpha_v\beta_8$, a cell adhesion molecule that binds LAP [81], causing a conformational change in latent TGF-β that enables its subsequent cleavage activation by matrix metalloproteinases [82]. Therefore, the perivascular co-location of megakaryocytes and Schwann cells may enable secretion of latent TGF-β by the former and subsequent TGF-β activation by the latter, a crucial duo to maintain HSCs in quiescence in perivascular niches (Figure 32.2). (Schwann cells were discovered by Theodor Schwann (1810–1882), a German physiologist.)

### 32.7.4 Adrenergic neurons

The bone marrow is innervated in particular with adrenergic nerves that secrete epinephrine and norepinephrine that bind to β2- and β3-adrenergic receptors expressed by MSCs in HSC niches. This β-adrenergic pathway regulates CXCL12 expression by MSCs as well as MSC quiescence and proliferation. Indeed, the β3-adrenergic pathway regulates the circadian oscillation of CXCL12 expression by MSCs and consequently circadian release of HSCs from bone marrow niches into the circulation in steady state [83]. In addition to this, both the β2- and β3-adrenergic pathways are necessary for maintaining MSCs and for functionality of HSC niches following cytotoxic injury. To demonstrate this, two models of *in vivo* ablation of peripheral adrenergic nerves were used: (1) chemical ablation of adrenergic nerves by injecting 6-hydroxy dopamine and (2) genetic ablation by injecting diphtheria toxin into *Th-Cre iDTR* mice in which Cre recombinase is driven by the promoter of the tyrosine hydroxylase gene. Tyrosine hydroxylase is the enzyme that converts tyrosine into L-dopamine and is specific for catecholaminergic nerves. In both models of adrenergic nerve ablation the recovery of nestin$^+$ MSCs and HSCs following cytotoxic injury with 5-fluorouracyl was compromised, leading to increased incidence of bone marrow failure and death [84]. Lucas *et al.* found that β2- and β3-adrenergic receptors present on MSCs block their proliferation and protect them from the cytotoxic effect of 5-fluorouracyl [84]. In the absence of adrenergic nerves, or following administration of β2- and β3-adrenergic antagonists, nestin$^+$ MSC proliferation was induced, resulting in increasing MSC sensitivity to cytotoxic drugs. Interestingly, cytotoxic drugs with peripheral neurotoxicity, such as cisplatin and vincristine, also killed adrenergic nerves in the bone marrow, sensitizing nestin$^+$ MSCs to subsequent irradiation or cytotoxic drug treatment. As a consequence of the enhanced destruction of MSCs, the number of HSCs surviving chemotherapy was significantly reduced [84]. Remarkably, administration of neuroprotective drugs, such as glial cell derived neurotrophic factor or 4-methylcatechol, prevented death of adrenergic nerves in response to cisplatin or vincristine, increasing MSC survival and hematopoietic recovery following treatment with these two drugs [84]. The clinical relevance of these findings is illustrated by the fact that cisplatin and vincristine are used to treat some types of lymphoma and acute leukemia. Up to 20% of patients so treated experience hematopoietic dysfunction and treatment-related mortality [85,86]. Therefore, adjuvant treatment with neuroprotective drugs may reduce the incidence of treatment-related bone marrow failure and the side effects and mortality in patients treated with cisplatin or vincristine.

### 32.7.5 Macrophages

Macrophages include a very diverse array of mononucleated myeloid cells with wide-ranging immune and homeostatic functions, from the phagocytosis and elimination of pathogens and tissue debris to the maintenance and homeostasis of a variety of tissues, such as the brain, liver, and lung [87]. Although monocytes are produced in the bone marrow from myeloid progenitors, the function of macrophages residing in the bone marrow is not fully understood. It has recently emerged that specific subsets of bone marrow macrophages are critical to the maintenance of HSC niches and osteoblasts in the bone marrow. Indeed, CD169$^+$ macrophages are found adjacent to nestin$^+$ MSCs in the mouse bone marrow [88]. Furthermore, a canopy of F4/80$^+$ macrophages lies just above osteoblasts at the endosteum [89,90]. Interestingly, *in vivo* ablation of these bone marrow macrophages by three independent methods, namely (1) the injection of G-CSF, (2) the administration of liposomes loaded with the cell-impermeable drug clodronate, and (3) genetic macrophage ablation using macrophage Fas-induced apoptosis, results in loss of osteoblasts, reduced expression of CXCL12 by MSCs, and induced mobilization of HSCs from their bone marrow niche into the circulation [88,90,91]. Therefore, together with adrenergic nerves, macrophages are key regulators of MSC function and HSC retention within their bone marrow niches. The mechanisms by which macrophages regulates MSCs remains unknown.

Studies in the mouse have revealed a complex picture of the HSC niche in which MSCs play a critical role by the secretion of factors essential to maintain HSC survival, quiescence, or proliferation (Figure 32.2). Although there is now consensus that many HSC niches and HSC-supportive MSCs are perivascular, whether they will promote quiescence or proliferation of HSCs depends on the balance between proliferative factors (such as E-selectin) and anti-proliferative factors (such as TGF-β or CXCL4), and which may ultimately depend on the location of these niches (peri-arteriolar or peri-sinusoidal) or the presence or absence of additional cells, such as endothelial cells, megakaryocytes, and Schwann cells (Figure 32.2).

**Table 32.3** Common features of mouse and human MSCs.

| | Mouse adult MSC | Human adult MSC |
|---|---|---|
| Antigen expression | | |
| Nestin | ✓ | ✓ |
| PDGFR | ✓ | ✓ |
| CD51 | ✓ | ? |
| NG2 | ✓ | ✓ |
| CD146 | ? | ✓ |
| CD45 | × | × |
| CD31 | × | × |
| CXCL12 | ✓ | ✓ |
| KIT ligand | ✓ | ✓ |
| Jagged-1 | ✓ | ✓ |
| Angiopoietin-1 | ✓ | ✓ |
| VCAM-1 | ✓ | ✓ |
| Clonal reconstitution of ossicles with BM *in vivo* | ✓ | ✓ |
| Support of HSC expansion | ✓ | ✓ |

## 32.8 Human mesenchymal stem cells and human hematopoiesis

Despite the complexity of HSC niches in the mouse bone marrow and the fact that exquisitely sensitive genetic studies with conditional gene induction or ablation *in vivo* in mice cannot be repeated in humans, progress has been made to better characterize genuine clonal multipotent MSCs and their HSC-supportive role in human bone marrow. There are similarities in marrow MSCs between mouse and man (Table 32.3).

In another seminal study, Sacchetti *et al.* compared the engraftment potential of cultured nonhematopoietic stromal cells derived from a variety of human tissues. In their model, plastic-adherent cells from two-dimensional cultures were harvested, cells adhered to a 3D support of hydroxyapatite/tricalcium phosphate matrix, and then packed in fibrin glue before subcutaneous implantation in immunodeficient mice. Eight weeks after implantation, mesenchymal cells isolated from the periosteum or from trabecular bones could form a compact ectopic bone but without a marrow cavity, whereas only stromal cells derived from whole human bone marrow could form an ectopic bone with a hematopoietic bone marrow inside it [92]. They further identified CD146, also called melanoma cell adhesion molecule or MUC18, as a marker of the bone marrow stromal cells able to develop an ectopic bone containing a bone marrow upon transplantation. In the human bone marrow, CD146 identifies cells that do not express hematopoietic or endothelial cell-specific molecules. This population of $CD146^+$ cells contains the whole CFU-F activity *in vitro* and contains complete potential to engraft in immunodeficient mice and forms an ectopic bone marrow, whereas $CD146^-$ cells have none of these abilities [92]. CFU-F derived from single $CD146^+$ cells can form ectopic ossicles with bone marrow upon implantation into mice. Furthermore, only $CD146^+$ cells at the single cell level could generate CFU-F that could serially replate to form secondary CFU-F. This suggested that $CD146^+$ cells can self-renew, at least *in vitro*, although *in vivo* self-renewal to serially regenerate ossicles and bone marrow was not demonstrated [92]. Nevertheless, these data are the first to pinpoint human mesenchymal cells from the bone marrow able to develop an ectopic bone containing a hematopoietic bone marrow upon transplantation *in vivo* and self-renew *in vitro*, thus fulfilling many attributes of MSCs capable of reforming an HSC niche upon transplantation in a recipient mouse. Like $nestin^+$ MSCs identified in the mouse bone marrow [34,38], human $CD146^+$ MSCs in the human bone marrow are perivascular and project long processes within the hematopoietic bone marrow tissue [92,93]. These $CD146^+$ cells also express PDGFR [92], nestin and LEPR [93], the HSC supportive chemokine CXCL12 [93], and HSC-supportive cytokines such as Jagged-1 and angiopoietin-1 [92] (Table 32.3). Although $CD146^+$ MSCs support the formation of an ectopic bone with bone marrow, the initial study from Sacchetti *et al.* did not show whether $CD146^+$ cell were capable of supporting HSCs directly. This was recently demonstrated using co-cultures of sorted $CD146^+$ cells and human $CD34^+$ HSPCs sorted from cord blood. Indeed, only $CD146^+$ cells could support the survival and maintenance of human HSCs capable of reconstituting human hematopoiesis upon transplantation in immunodeficient mice. After 2 weeks of co-culture without additional exogenous growth factors, hematopoietic cells co-cultured with $CD146^+$ MSCs were still capable of engrafting and generating human B lymphocytes and monocytes upon transplantation in immunodeficient mice, whereas cells recovered from co-cultures of HSPCs with nonsorted plastic-adherent bone marrow stromal cells were incapable of hematopoietic reconstitution upon transplantation [93]. Therefore, taken together, these two studies demonstrate that $CD146^+$ cells isolated from the human bone marrow are MSCs capable of regenerating an ectopic bone containing a bone marrow with HSC niches and directly supporting HSC maintenance [92–94].

Of note is that perivascular $CD146^+$ cells are not exclusively found in the bone marrow as they have been identified in, and isolated from, human adipose tissue, skeletal muscle, pancreas, and placenta [93,95]. Similar to $CD146^+$ MSCs isolated from the bone marrow, these MSCs isolated from alternative tissues were also perivascular and could support the formation of ectopic bones containing a hematopoietic bone marrow upon transplantation into immunodeficient mice [93,95].

While the direct role of human $CD146^+$ MSCs in regenerating a human bone containing a hematopoietic bone marrow and directly supporting human HSCs is now well documented, the potential roles of alternative niche cells have not been extensively

studied in human bone marrow. In the case of endothelial cells there is indirect evidence that human endothelial cells may also support human HSCs. However, the evidence is indirect because it is very difficult to culture bone marrow endothelial cells. Human umbilical vein endothelial cells can be immortalized following transfection with the E4ORF1 gene of adenoviruses, which enables long-term culture of these endothelial cells in serum-free medium without addition of exogenous angiogenic factors [18]. Compared with monocultures of human cord blood $CD34^+$ HSPCs with purified recombinant human kit ligand, thrombopoietin, and Flt-3 ligand, co-cultures of human HSPCs with this cytokine supplement together with immortalized human endothelial cells supported a much greater expansion of phenotypic human HSCs ($Lin^-$ $CD34^+$ $CD45RA^-$ $CD49f^+$) and greater expansion of HSCs able to reconstitute human hematopoiesis upon transplantation into immunodeficient mice [18], demonstrating that immortalized human endothelial cells can directly support human HSCs. The mechanisms by which immortalized human endothelial cells support HSCs has, however, not been reported.

## 32.9 Conclusions

Recent experimental evidence demonstrates that clonal multipotent MSCs in the adult bone marrow directly support HSCs in mice and humans and are capable of reforming a bone containing a functional hematopoietic bone marrow when transplanted onto an appropriate scaffold to support ectopic bone formation. Although the ontogeny of MSCs in the bone marrow is complex and may undergo several waves [50,51], MSCs in the adult bone marrow are perivascular, express nestin, and produce HSC-supportive chemokines and growth factors in both humans and mice (Table 32.3). It is also clear that MSCs are not alone in regulating HSCs in bone marrow niches but act in concert with endothelial cells, and in the mouse with megakaryocytes, Schwann cells, and macrophages (Figure 32.2). Elucidation of the many mechanisms deployed by MSCs and other niche cells to regulate HSCs in the bone marrow enables the design of novel therapeutic strategies specifically targeting these HSC–niche interactions to improve therapeutic outcomes. Several of these "nichotherapies" already exist [96]. For instance, G-CSF mobilizes HSCs from the bone marrow into the circulation by inhibiting macrophages that regulate HSC niches, causing the downregulation of CXCL12 and VCAM-1 [88,90,91], two key proteins expressed by mesenchymal progenitor cells and endothelial cells to retain HSCs within the bone marrow. G-CSF also causes a reduction of oxygen availability in the bone marrow, which contributes to HSC mobilization [97,98]. These discoveries have enabled the development of drugs targeting these niche interactions (also called nichotherapies), such as CXCR4 inhibitors [99–101] and prolylhydoxylase inhibitors [98] to further boost HSC mobilization for transplantation, particularly in patients undergoing autologous transplantation after prior successive rounds of chemotherapy that often compromise subsequent HSC mobilization and collection for transplantation [102]. Furthermore, it has recently emerged that leukemia-initiating cells, which represent a small subset of leukemia cells that self-renew and clonally reinitiate the leukemia [103,104], hijack HSC niches in the leukemic bone marrow where they are sheltered and protected from the cytotoxic effects of chemotherapy [105,106]. The poor response of leukemia-initiating cells to cytoxic chemotherapy and tyrosine kinase inhibitors can lead to recurrence of the leukemia once the chemotherapy regimen has ended. Hence, the concept of dislodging chemotherapy-resistant leukemia-initiating cells from their chemoprotective niches by mobilizing them with CXCR4 antagonists prior to chemotherapy in order to sensitize them to chemotherapies has emerged. This approach has been successfully employed in mice to sensitize acute myeloid leukemia-initiating cells to the chemotherapeutic agents cytosine arabinoside and doxorubicin [107,108], reduce leukemia burden, and increase mouse survival [107,108]. Likewise, L-ribose-based L-RNA aptamers (called spiegelmers because L-ribose L-RNA are mirror-image enantiomers of naturally occurring D-RNA based on D-ribose – *spiegel* is German for mirror) specifically block CXCL12 and have been reported to mobilize and chemosentitize mouse and human multiple myeloma cells *in vivo* [109]. These encouraging results in the mouse have prompted the initiation of approximately a dozen clinical trials to test the efficacy of CXCR4 inhibitors in increasing the sensitivity of malignant cells to chemotherapy treatment.

## References


1 A. Wilson, E. Laurenti, G. Oser, *et al.* Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair. *Cell* 2008;**135**:1118–1129.

2 S. N. Catlin, L. Busque, R. E. Gale, *et al.* The replication rate of human hematopoietic stem cells *in vivo*. *Blood* 2011;**117**:4460–4466.

3 S. Bekkering, R. Torensma. Another look at the life of a neurtrophil. *World J Hematol* 2013;**2**:44–58.

4 J. Sun, A. Ramos, B. Chapman, *et al.* Clonal dynamics of native haematopoiesis. *Nature* 2014;**514**:322–327.

5 M. A. G. Essers, S. Offner, W. E. Blanco-Bose, *et al.* IFNα activates dormant haematopoietic stem cells *in vivo*. *Nature* 2009;**458**:904–908.

6 M. T. Baldridge, K. Y. King, N. C. Boles, *et al.* Quiescent haematopoietic stem cells are activated by IFN-γ in response to chronic infection. *Nature* 2010;**465**:793–797.

7 O. J. Borge, V. Ramsfjell, L. Cui, S. E. Jacobsen. Ability of early acting cytokines to directly promote survival and suppress apoptosis of human primitive $CD34^+CD38^-$ bone marrow cells with multilineage potential at the single-cell level: key role of thrombopoietin. *Blood* 1997;**90**:2282–2292.

8 D. N. Haylock, M. J. Horsfall, T. L. Dowse, *et al.* Increased recruitment of hematopoietic progenitor cells underlies the *ex vivo* expansion potential of FLT3 ligand. *Blood* 1997;**90**:2260–2272.

9 D. N. Haylock, S. K. Nilsson. Expansion of umbilical cord blood for clinical transplantation. *Curr Stem Cell Res Ther* 2007;**2**:324–335.

10 H. M. Prince, P. J. Simmons, G. Whitty, *et al.* Improved haematopoietic recovery following transplantation with *ex vivo*-expanded mobilized blood cells. *Br J Haematol* 2004;**126**:536–545.

11 E. L. Kittler, S. O. Peters, R. B. Crittenden, *et al.* Cytokine-facilitated transduction leads to low-level engraftment in nonablated hosts. *Blood* 1997;**90**:865–872.

12 S. O. Peters, E. L. Kittler, H. S. Ramshaw, P. J. Quesenberry. *Ex vivo* expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. *Blood* 1996;**87**:30–37.

13 V. I. Rebel, M. Tanaka, J. S. Lee, *et al.* One-day *ex vivo* culture allows effective gene transfer into human nonobese diabetic/severe combined immune-deficient repopulating cells using high-titer vesicular stomatitis virus G protein pseudotyped retrovirus. *Blood* 1999;**93**:2217–2224.

14 A. Gothot, J. C. van der Loo, D. W. Clapp, E. F. Srour. Cell cycle-related changes in repopulating capacity of human mobilized peripheral blood $CD34^+$ cells in non-obese diabetic/severe combined immune-deficient mice. *Blood* 1998;**92**:2641–2649.

15 L. E. Purton, I. D. Bernstein, S. J. Collins. All-trans retinoic acid enhances the long-term repopulating activity of cultured hematopoietic stem cells. *Blood* 2000;**95**:470–477.

16 T. Eto, I. Winkler, L. E. Purton, J. P. Lévesque. Contrasting effects of P-selectin and E-selectin on the differentiation of murine hematopoietic progenitor cells. *Exp Hematol* 2005;**33**:232–242.

17 M. de Lima, I. McNiece, S. N. Robinson, *et al.* Cord-blood engraftment with *ex vivo* mesenchymal-cell coculture. *N Engl J Med* 2012;**367**:2305–2315.

18 J. M. Butler, E. J. Gars, D. J. James, *et al.* Development of a vascular niche platform for expansion of repopulating human cord blood stem and progenitor cells. *Blood* 2012;**120**:1344–1347.

19 J. M. Butler, D. J. Nolan, E. L. Vertes, *et al.* Endothelial cells are essential for the self-renewal and repopulation of Notch-dependent hematopoietic stem cells. *Cell Stem Cell* 2010;**6**:251–264.

20 M. Tavassoli, W. H. Crosby. Transplantation of marrow to extramedullary sites. *Science* 1968;**161**:54–56.

21 T. Takaku, D. Malide, J. Chen, *et al.* Hematopoiesis in 3 dimensions: human and murine bone marrow architecture visualized by confocal microscopy. *Blood* 2010;**116**:e41–e55.

22 J. P. Lévesque, I. G. Winkler. Hierarchy of immature hematopoietic cells related to blood flow and niche. *Curr Opin Hematol* 2011;**18**:220–225.

23 C. Nombela-Arrieta, G. Pivarnik, B. Winkel, *et al.* Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. *Nat Cell Biol* 2013;**15**:533–543.

24 J. A. Spencer, F. Ferraro, E. Roussakis, *et al.* Direct measurement of local oxygen concentration in the bone marrow of live animals. *Nature* 2014;**508**:269–273.

25 I. G. Winkler, V. Barbier, R. Wadley, *et al.* Positioning of bone marrow hematopoietic and stromal cells relative to blood flow *in vivo*: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. *Blood* 2010;**116**:375–385.

26 R. Schofield. The relationship between the spleen colony-forming cell and the haemopoietic stem cell. *Blood Cells* 1978;**4**:7–25.

27 P. Bianco, X. Cao, P. S. Frenette, *et al.* The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine. *Nat Med* 2013;**19**:35–42.

28 H. Castro-Malaspina, R. E. Gay, G. Resnick, *et al.* Characterization of human bone marrow fibroblast colony-forming cells (CFU-F) and their progeny. *Blood* 1980;**56**:289–301.

29 A. J. Friedenstein, J. F. Gorskaja, N. N. Kulagina. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp Hematol* 1976;**4**:267–274.

30 S. Gronthos, A. C. Zannettino, S. J. Hay, *et al.* Molecular and cellular characterisation of highly purified stromal stem cells derived from human bone marrow. *J Cell Sci* 2003;**116**:1827–1835.

31 S. Shi, S. Gronthos. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. *J Bone Miner Res* 2003;**18**:696–704.

32 L. Zimmerman, B. Parr, U. Lendahl, *et al.* Independent regulatory elements in the nestin gene direct transgene expression to neural stem cells or muscle precursors. *Neuron* 1994;**12**:11–24.

33 U. Lendahl, L. B. Zimmerman, R. D. McKay. CNS stem cells express a new class of intermediate filament protein. *Cell* 1990;**60**:585–595.

34 S. Mendez-Ferrer, T. V. Michurina, F. Ferraro, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

35 M. J. Kiel, O. H. Yilmaz, T. Iwashita, *et al.* SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 2005;**121**:1109–1121.

36 J. P. Lévesque, F. M. Helwani, I. G. Winkler. The endosteal "osteoblastic" niche and its role in hematopoietic stem cell homing and mobilization. *Leukemia* 2010;**24**:1979–1992.

37 J. G. Naglich, J. E. Metherall, D. W. Russell, L. Eidels. Expression cloning of a diphtheria toxin receptor: identity with a heparin-binding EGF-like growth factor precursor. *Cell* 1992;**69**:1051–1061.

38 Y. Kunisaki, I. Bruns, C. Scheiermann, *et al.* Arteriolar niches maintain haematopoietic stem cell quiescence. *Nature* 2013;**502**:637–643.

39 A. Wilson, A. Trumpp Bone-marrow haematopoietic-stem-cell niches. *Nat Rev Immunol* 2006;**6**:93–106.

40 I. G. Winkler, V. Barbier, B. Nowlan, *et al.* Vascular niche E-selectin regulates hematopoietic stem cell dormancy, self renewal and chemoresistance. *Nat Med* 2012;**18**:1651–1657.

41 T. Suda, K. Takubo, G. L. Semenza. Metabolic regulation of hematopoietic stem cells in the hypoxic niche. *Cell Stem Cell* 2011;**9**:298–310.

42 K. Takubo, N. Goda, W. Yamada, *et al.* Regulation of the HIF-1α level is essential for hematopoietic stem cells. *Cell Stem Cell* 2010;**7**:391–402.

43 C. E. Forristal, I. G. Winkler, B. Nowlan, *et al.* Pharmacologic stabilization of HIF-1α increases hematopoietic stem cell quiescence *in vivo* and accelerates blood recovery after severe irradiation. *Blood* 2013;**121**:759–769.

44 G. B. Bradford, B. Williams, R. Rossi, *et al.* Quiescence, cycling, and turnover in the primitive hematopoietic stem cell compartment. *Exp Hematol* 1997;**25**:445–453.

45 L. Ding, T. L. Saunders, G. Enikolopov, S. J. Morrison. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature* 2012;**481**:457–462.

46 S. Pinho, J. Lacombe, M. Hanoun, *et al.* PDGFRα and CD51 mark human $nestin^+$ sphere-forming mesenchymal stem cells capable of hematopoietic progenitor cell expansion. *J Exp Med* 2013;**210**:1351–1367.

47 K. M. Zsebo, D. A. Williams, E. N. Geissler, *et al.* Stem cell factor is encoded at the Sl locus of the mouse and is the ligand for the c-kit tyrosine kinase receptor. *Cell* 1990;**63**:213–224.

48 H. E. Broxmeyer, R. Maze, K. Miyazawa, *et al.* The kit receptor and its ligand, steel factor, as regulators of hemopoiesis. *Cancer Cells* 1991;**3**:480–487.

49 M. Ogawa, Y. Matsuzaki, S. Nishikawa, *et al.* Expression and function of c-kit in hemopoietic progenitor cells. *J Exp Med* 1991;**174**:63–71.

50 N. Ono, W. Ono, T. Mizoguchi, *et al.* Vasculature-associated cells expressing nestin in developing bones encompass early cells in the osteoblast and endothelial lineage. *Dev Cell* 2014;**29**:330–339.

51 T. Mizoguchi, S. Pinho, J. Ahmed, *et al.* Osterix marks distinct waves of primitive and definitive stromal progenitors during bone marrow development. *Dev Cell* 2014;**29**:340–349.

52 M. Logan, J. F. Martin, A. Nagy, *et al.* Expression of Cre recombinase in the developing mouse limb bud driven by a Prxl enhancer. *Genesis* 2002;**33**:77–80.

53 S. Provot, D. Zinyk, Y. Gunes, *et al.* Hif-1α regulates differentiation of limb bud mesenchyme and joint development. *J Cell Biol* 2007;**177**:451–464.

54 A. Greenbaum, Y.-M. S. Hsu, R. B. Day, *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature* 2013;**495**:227–230.

55 B. I. Lord, N. G. Testa, J. H. Hendry. The relative spatial distributions of CFUs and CFUc in the normal mouse femur. *Blood* 1975;**46**:65–72.

56 S. K. Nilsson, H. M. Johnston, J. A. Coverdale. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. *Blood* 2001;**97**:2293–2299.

57 Y. Xie, T. Yin, W. Wiegraebe, *et al.* Detection of functional haematopoietic stem cell niche using real-time imaging. *Nature* 2009;**457**:97–101.

58 J. Zhang, C. Niu, L. Ye, *et al.* Identification of the haematopoietic stem cell niche and control of the niche size. *Nature* 2003;**425**:836–841.

59 L. M. Calvi, N. A. Sims, J. L. Hunzelman, *et al.* Activated parathyroid hormone/parathyroid hormone-related protein receptor in osteoblastic cells differentially affects cortical and trabecular bone. *J Clin Invest* 2001;**107**:277–286.

60 D. Visnjic, Z. Kalajzic, D. W. Rowe, *et al.* Hematopoiesis is severely altered in mice with an induced osteoblast deficiency. *Blood* 2004;**103**:3258–3264.

61 J. Zhu, R. Garrett, Y. Jung, *et al.* Osteoblasts support B-lymphocyte commitment and differentiation from hematopoietic stem cells. *Blood* 2007;**109**:3706–3712.

62 T. Sugiyama, H. Kohara, M. Noda, T. Nagasawa. Maintenance of the hematopoietic stem cell pool by CXCL12–CXCR4 chemokine signaling in bone marrow stromal cell niches. *Immunity* 2006;**25**:977–988.

63 L. Ding, S. J. Morrison. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature* 2013;**495**:231–235.

64 I. G. Winkler, L. J. Bendall, C. E. Forristal, *et al.* B-lymphopoiesis is stopped by mobilizing doses of G-CSF and is rescued by overexpression of the anti-apoptotic protein Bcl2. *Haematologica* 2013;**98**:325–333.

65 M. H. G. P. Raaijmakers, S. Mukherjee, S. Guo, *et al.* Bone progenitor dysfunction induces myelodysplasia and secondary leukaemia. *Nature* 2010;**464**:852–857.

66 J. P. Lévesque, J. Hendy, Y. Takamatsu, *et al.* Disruption of the CXCR4/CXCL12 chemotactic interaction during hematopoietic stem cell mobilization induced by GCSF or cyclophosphamide. *J Clin Invest* 2003;**111**:187–196.

67 I. Petit, M. Szyper-Kravitz, A. Nagler, *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 2002;**3**:687–694.

68 J. P. Lévesque, Y. Takamatsu, S. K. Nilsson, *et al.* Vascular cell adhesion molecule-1 (CD106) is cleaved by neutrophil proteases in the bone marrow following hematopoietic progenitor cell mobilization by granulocyte colony-stimulating factor. *Blood* 2001;**98**:1289–1297.

69 T. Ulyanova, L. M. Scott, G. V. Priestley, *et al.* VCAM-1 expression in adult hematopoietic and nonhematopoietic cells is controlled by tissue-inductive signals and reflects their developmental origin. *Blood* 2005;**106**:86–94.

70 M. G. Poulos, P. Guo, N. M. Kofler, *et al.* Endothelial Jagged-1 is necessary for homeostatic and regenerative hematopoiesis. *Cell Rep* 2013;**4**:1022–1034.

71 N. O. Fortunel, A. Hatzfeld, J. A. Hatzfeld. Transforming growth factor-β: pleiotropic role in the regulation of hematopoiesis. *Blood* 2000;**96**:2022–2036.

72 J. Hatzfeld, M. L. Li, E. L. Brown, *et al.* Release of early human hematopoietic progenitors from quiescence by antisense transforming growth factor beta 1 or Rb oligonucleotides. *J Exp Med* 1991;**174**:925–929.

73 S. Yamazaki, A. Iwama, S.-i. Takayanagi, *et al.* TGF-β as a candidate bone marrow niche signal to induce hematopoietic stem cell hibernation. *Blood* 2009;**113**:1250–1256.

74 U. Blank, G. Karlsson, J. L. Moody, *et al.* Smad7 promotes self-renewal of hematopoietic stem cells. *Blood* 2006;**108**:4246–4254.

75 G. Karlsson, U. Blank, J. L. Moody, *et al.* Smad4 is critical for self-renewal of hematopoietic stem cells. *J Exp Med* 2007;**204**:467–474.

76 T. Junt, H. Schulze, Z. Chen, *et al.* Dynamic visualization of thrombopoiesis within bone marrow. *Science* 2007;**317**:1767–1770.

77 N. L. Thompson, K. C. Flanders, J. M. Smith, *et al.* Expression of transforming growth factor-beta 1 in specific cells and tissues of adult and neonatal mice. *J Cell Biol* 1989;**108**:661–669.

78 S. Y. Heazlewood, R. J. Neaves, B. Williams, *et al.* Megakaryocytes co-localise with hemopoietic stem cells and release cytokines that up-regulate stem cell proliferation. *Stem Cell Res* 2013;**11**:782–792.

79 I. Bruns, D. Lucas, S. Pinho, *et al.* Megakaryocytes regulate hematopoietic stem cell quiescence through CXCL4 secretion. *Nat Med* 2014;**20**:1315–1320.

80 M. Zhao, J. M. Perry, H. Marshall, *et al.* Megakaryocytes maintain homeostatic quiescence and promote post-injury regeneration of hematopoietic stem cells. *Nat Med* 2014;**20**:1321–1326.

81 S. Yamazaki, H. Ema, G. Karlsson, *et al.* Nonmyelinating Schwann cells maintain hematopoietic stem cell hibernation in the bone narrow niche. *Cell* 2011;**147**:1146–1158.

82 D. Mu, S. Cambier, L. Fjellbirkeland, *et al.* The integrin $\alpha_v\beta_8$ mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-β1. *J Cell Biol* 2002;**157**:493–507.

83 S. Mendez-Ferrer, D. Lucas, M. Battista, P. S. Frenette. Haematopoietic stem cell release is regulated by circadian oscillations. *Nature* 2008;**452**:442–447.

84 D. Lucas, C. Scheiermann, A. Chow, *et al.* Chemotherapy-induced bone marrow nerve injury impairs hematopoietic regeneration. *Nat Med* 2013;**19**:695–703.

85 A. H. Elmaagacli, M. Koldehoff, N. K. Steckel, *et al.* Cytochrome P450 2C19 loss-of-function polymorphism is associated with an increased treatment-related mortality in patients undergoing allogeneic transplantation. *Bone Marrow Transplant* 2007; **40**:659–664.

86 J.-P. Levesque, I. G. Winkler. It takes nerves to recover from chemotherapy. *Nat Med* 2013;**19**:669–671.

87 A. Sica, A. Mantovani. Macrophage plasticity and polarization: *in vivo veritas*. *J Clin Invest* 2012;**122**:787–795.

88 A. Chow, D. Lucas, A. Hidalgo, *et al.* Bone marrow $CD169^{+}$ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. *J Exp Med* 2011;**208**:261–271.

89 M. K. Chang, L.-J. Raggatt, K. A. Alexander, *et al.* Osteal tissue macrophages are intercalated throughout human and mouse bone lining tissues and regulate osteoblast function *in vitro* and *in vivo*. *J Immunol* 2008;**181**:1232–1244.

90 I. G. Winkler, N. A. Sims, A. R. Pettit, *et al.* Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. *Blood* 2010;**116**:4815–4828.

91 M. J. Christopher, M. Rao, F. Liu, *et al.* Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. *J Exp Med* 2011; **208**:251–260.

92 B. Sacchetti, A. Funari, S. Michienzi, *et al.* Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–336.

93 M. Corselli, C. J. Chin, C. Parekh, *et al.* Perivascular support of human hematopoietic stem/progenitor cells. *Blood* 2013;**121**: 2891–2901.

94 J.-P. Levesque. A niche in a dish: pericytes support HSC. *Blood* 2013;**121**:2816–2818.

95 M. Crisan, S. Yap, L. Casteilla, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.

96 J.-P. Levesque, I. G. Winkler, J. E. J. Rasko. Nichotherapy for stem cells: There goes the neighborhood. *Bioessays* 2013;**35**:183–190.

97 J.-P. Lévesque, I. G. Winkler, J. Hendy, *et al.* Hematopoietic progenitor cell mobilization results in hypoxia with increased hypoxia-inducible transcription factor-1a and vascular endothelial growth factor A in bone marrow. *Stem Cells* 2007;**25**:1954–1965.

98 C. E. Forristal, B. Nowlan, V. Barbier, *et al.* FG-4497, a pharmacological stabilizer of HIF-1α protein, synergistically enhances hematopoietic stem cells (HSC) mobilization in response to G-CSF and plerixafor. *Blood* 2012;**120**:216 [Abstract].

99 H. E. Broxmeyer, C. M. Orschell, D. W. Clapp, *et al.* Rapid mobilization of murine and human hematopoietic stem and progenitor cells with AMD3100, a CXCR4 antagonist. *J Exp Med* 2005;**201**:1307–1318.

100 J. F. DiPersio, I. N. Micallef, P. J. Stiff, *et al.* Phase III prospective randomized double-blind placebo-controlled trial of plerixafor plus granulocyte colony-stimulating factor compared with placebo plus granulocyte colony-stimulating factor for autologous stem-cell mobilization and transplantation for patients with non-Hodgkin's lymphoma. *J Clin Oncol* 2009;**27**:4767–4773.

101 D. Karpova, K. Dauber, G. Spohn, *et al.* The novel CXCR4 antagonist POL5551 mobilizes hematopoietic stem and progenitor cells with greater efficiency than plerixafor. *Leukemia* 2013;**27**:2322–2331.

102 L. B. To, J.-P. Levesque, K. E. Herbert. How I treat patients who mobilize hematopoietic stem cells poorly. *Blood* 2011;**118**: 4530–4540.

103 D. Bonnet, J. E. Dick. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997;**3**:730–737.

104 J. A. Magee, E. Piskounova, S. J. Morrison. Cancer stem cells: impact, heterogeneity, and uncertainty. *Cancer Cell* 2012;**21**: 283–2896.

105 F. Ishikawa, S. Yoshida, Y. Saito, *et al.* Chemotherapy-resistant human AML stem cells home to and engraft within the bone-marrow endosteal region. *Nat Biotechnol* 2007;**25**:1315–1321.

106 M. Ninomiya, A. Abe, A. Katsumi, *et al.* Homing, proliferation and survival sites of human leukemia cells *in vivo* in immunodeficient mice. *Leukemia* 2006;**21**:136–142.

107 B. Nervi, P. Ramirez, M. P. Rettig, *et al.* Chemosensitization of acute myeloid leukemia (AML) following mobilization by the CXCR4 antagonist AMD3100. *Blood* 2009;**113**:6206–6214.

108 Z. Zeng, Y. Xi Shi, I. J. Samudio, *et al.* Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood* 2009;**113**: 6215–6224.

109 A. M. Roccaro, A. Sacco, W. G. Purschke, *et al.* SDF-1 inhibition targets the bone marrow niche for cancer therapy. *Cell Rep* 2014;**9**:118–128.

CHAPTER 33

# The modulatory effects of mesenchymal stromal cells on the innate immune system

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## 33.1 Introduction to the innate immune system

The innate immune system is broadly present in all organisms, including both plant and animal species. Also known as the nonspecific immune system, components of innate immunity are responsible for the initial immunological response toward pathogens and foreign material, which is usually in the form of an immediate and antigen-nonspecific response. In contrast to the evolutionarily younger adaptive immune system, this "first line" defense system does not confer long-lasting, protective immunity for the host. However, specific cell types within the innate immune system are responsible for activation of the adaptive immune system, and thus cells of this system also act as the bridge to link the two arms of the immune response together.

Despite the universality of the innate immune system, research on mesenchymal stem/stromal cell (MSC) interactions with this more ancient arm of the immune system has lagged behind that of MSCs and adaptive immune cells. Moreover, the largest proportion of reports of innate immune interactions with MSCs centers on dendritic cells (DCs), whose effector outcomes affect the adaptive immune system, especially T cell responses. Increasingly, however, targeting of the innate immune system has yielded new and effective treatments toward inflammatory diseases and conditions, thus shifting interest into further understanding of the myriad of leukocytes within this branch of the immune system [1]. It is anticipated, therefore, that insights into MSC interactions with the wide range of innate immune cells will be rapidly forthcoming.

A schema illustrating the immunomodulatory mechanisms of MSCs on the innate immune system is shown in Figure 33.1.

## 33.2 Interactions with dendritic cells

DCs are the quintessential antigen-presenting cell (APC) and considered the sentinels of the immune system. Derived from both myeloid and lymphoid precursors, DCs phagocytose pathogens and then present components of these pathogens as antigens to T lymphocytes, which then leads to lymphocyte activation. These key regulators also secrete cytokines to activate natural killer (NK) lymphocytes. Thus, DCs are messengers of the immune system and activate both adaptive and innate immune responses. Even more remarkably, DCs can fine-tune the immune response from being robustly immunogenic to being frankly tolerogenic. This depends on many parameters, including their own differentiation status [2]. Given the crucial role of DCs in the immune system, it is not surprising that the majority of publications of MSC interactions with innate leukocytes are focused on these leukocytes.

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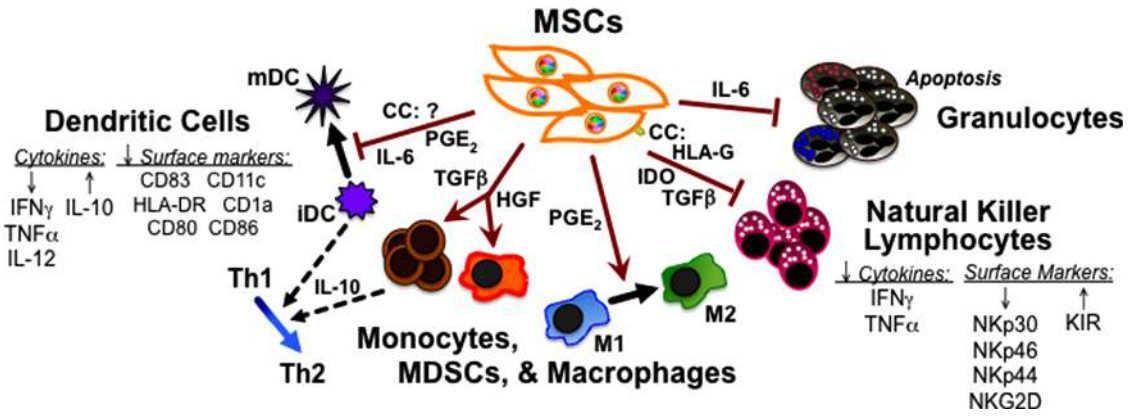



**Figure 33.1** Immunomodulatory mechanisms of MSCs on innate leukocytes. CC: cell contact; mDC: mature dendritic cell; iDC: immature dendritic cell; IL: interleukin; $PGE_2$: prostaglandin $E_2$; IFN-γ: interferon-γ; TGF-β: transforming growth factor-β; TNF-α: tumor necrosis factor-α; HLA-DR: human leukocyte antigen-DR; HGF: hepatocyte growth factor; Th: T helper; MDSCs: myeloid-derived suppressor cells; IDO: indoleamine 2,3-dioxyenase; KIR: killer inhibitory receptor; HLA-G: human leukocyte antigen-G. (See color plate section.)

In general, reports show that MSCs inhibit the immunostimulatory capacity of DCs, inducing DCs into more tolerogenic phenotypes either by blocking differentiation or through other mechanisms. Co-culture of MSCs with monocytes, the precursors of DCs in the peripheral blood, or of bone marrow hematopoietic stem cells in the presences of cytokines and factors to induce differentiation into DCs, generally appears to halt the process, so that DCs remain in a more immature and immunoregulatory state [3–5]. DCs co-cultured with MSCs show reduced surface expression of a number of maturation markers, including CD83, HLA-DR, CD1a, CD11c, and the costimulatory molecules CD80 and CD86 [3,6–9], and may even generate novel populations of regulatory DCs [7]. Moreover, MSCs modulate DCs to decrease secretion of many proinflammatory cytokines, including TNF-α, IFN-γ, and IL-12 [3,8–11], while increasing secretion of immunomodulatory cytokines such as IL-10 [8,9,11,12]. Interestingly, DCs co-cultured with MSCs show enhanced endocytic ability, which paradoxically does not translate to increased functional capacity [6,7,9]. The effects of MSCs on DCs appear to be largely mediated by MSC-secreted paracrine factors [4,13], the most consistent being $PGE_2$ [13,14] and IL-6 [8,14,15], but there is some evidence that cell–cell contact may at least play a partial role in these processes [5,7,15]. The ability of DCs to home to regional lymph nodes is also decreased by the administration of MSCs as demonstrated in *in vivo* studies. In a mouse model of acute graft-versus-host disease, infusion of MSCs delayed disease onset by decreasing the ability of DCs to home to secondary lymphoid organs [16].

Given the known role of DCs in T lymphocyte activation, it is not surprising that at least some of the immunomodulatory effects of MSCs on T cells appear to require or at least involve DCs [17]. A number of reports show that MSC suppression of T cell proliferation is through the modulation of mature DCs by MSCs to more immature tolerogenic phenotypes, thereby decreasing the capacity to activate T cells [3,4,8,9,11,13]. This results in the alteration of the cytokine milieu from a Th1 into a Th2 axis [6,8], as well as the expansion of regulatory T cells (Tregs), a population of immunomodulatory $CD4^+$ T lymphocytes [8,11,18]. These *in vitro* findings are corroborated by *in vivo* studies in which infusion of MSCs affects the capacity of DCs to prime T cells by blocking DC homing to draining lymph nodes [19].

Remarkably, adult bone marrow MSCs have been shown to harbor functions similar to the antigen presentation function of DCs. It has been shown that IFN-γ-stimulated bone marrow MSCs can present exogenous antigens through the upregulation of surface major histocompatibility class (MHC) II molecules, which then induce $CD4^+$ T cell activation [20–22]. Moreover, adult bone marrow MSCs can also cross-present exogenous antigens to activate $CD8^+$ T cells [23,24]. These results differ from those of fetal-source MSCs, which express low or limited levels of MHC II molecules even after IFN-γ stimulation, rendering these MSCs poor APCs [25–27].

Increasingly more reports demonstrate that MSCs express high levels of toll-like receptors (TLRs), including TLR1, TLR3, TLR4, and TLR5. Highly expressed on APCs, TLRs are receptors that recognize conserved pathogen-derived components found on microbial pathogens. Triggering of TLR3 (which binds double-stranded RNA) and TLR4 (which binds lipopolysaccharide) on bone marrow and nasal MSCs has been reported to lead to a more proinflammatory phenotype inducing production of cytokines such as IL-1β, IL-6, and IL-8 [23,28–32]. Conversely, others have reported that triggering of TLR on bone marrow MSCs actually increases downmodulation of immunity through the induction of indoleamine 3,4-dioxygenase-1 (IDO1) and production of immunosuppressive kynurenines [33]. Even more interesting are reports showing induction of either a proinflammatory or an immunosuppressive phenotype in bone marrow and umbilical cord MSCs depending on the specific TLR stimulated [34–37]. These conflicting findings may be due to tissue-specific differences of MSCs [38], heterogeneity due to differences in isolation methods [39], and differences in

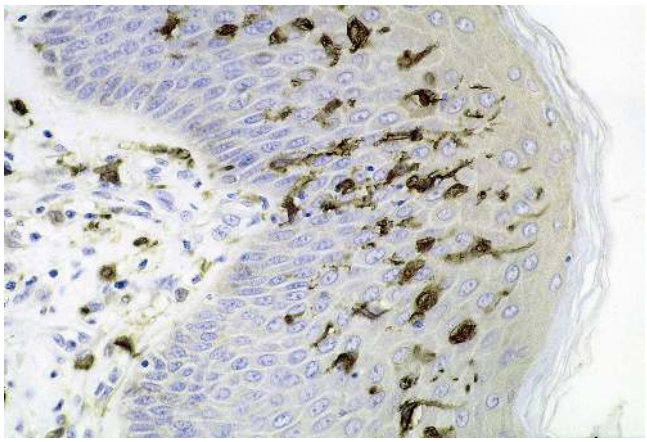


**Figure 33.2** Section of skin showing large numbers of dendritic (Langerhans) cells stained with S100 immunoperoxidase stain in the epidermis of a person with *Mycobacterium ulcerans* infection. *M. ulcerans* is a slow-growing mycobacterium that classically infects the skin and subcutaneous tissues, giving rise to indolent nonulcerated nodules and plaques and ulcerated lesions. The latter are known as Buruli or Bairnsdale ulcers. The Langerhans cell is named after Paul Langerhans, a German physician and anatomist, who discovered the cells at the age of 21 while he was a medical student. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Dendritic_cells.jpg#/media/File:Dendritic_cells.jpg. (See color plate section.)

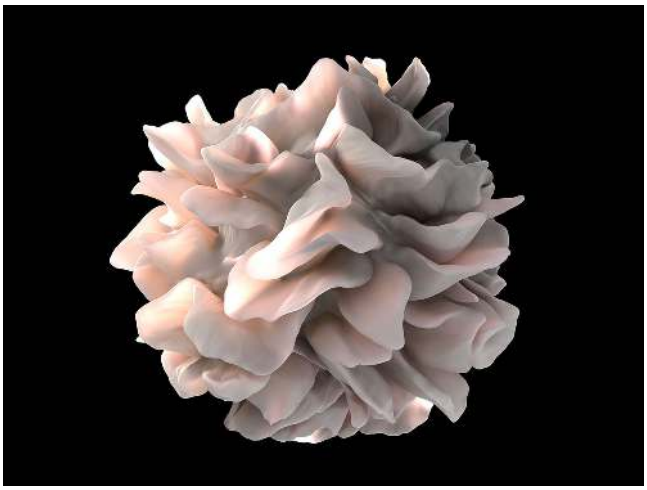


**Figure 33.3** Ion-abrasion scanning electron micrograph of a DC. Artistic rendering of the surface of a human dendritic cell illustrating the unexpected discovery of sheet-like processes that fold back onto the membrane surface. When exposed to viruses, for example, these sheets entrap them in the vicinity and focus them to contact zones with T cells targeted for infection. These studies were carried out using ion-abrasion scanning electron microscopy, a new technology developed and applied for three-dimensional cellular imaging. (See color plate section.) *Source:* dendritic cell revealed by National Institutes of Health (NIH). (See color plate section.)

the stimulator used, including dose and timing. More comparative studies on tissue-specific MSCs, better standardization of MSCs, and more concise experimental descriptions are necessary for a clear understanding of the role of TLRs in MSC immunomodulation and biology.

Figure 33.2 shows an infiltrate of DCs in the skin (where they are known as Langerhans cells) in a person with *Mycobacterium ulcerans* infection. Figure 33.3 shows the astonishing appearance of DCs obtained by ion-abrasion scanning electron microscopy.

## 33.3 Interactions with monocytes, macrophages, and immature myeloid cells

Approximately 10–15% of circulating leukocytes in human peripheral blood consist of monoyctes. Best known for being the precursor cells for macrophages and DCs, the effector function of monocytes have only recently been reported [40]. Correspondingly, while there are relatively more reports on interactions of MSCs with monocyte progenitors of DCs and macrophages, reports of monocyte and MSC interactions have lagged behind. One of the first reports in the MSC immunomodulation literature to mention monocyte interactions was with regard to T cell responses, in which monocytes were found to secrete IL-1β and induce MSCs to produce TGF-β, thereby suppressing T cell responses [41]. Further reports confirmed that monocytes often act as an intermediary in MSC modulation of T cell responses [42,43]. Interactions between MSCs and monocytes appear to be mediated by soluble factors, including MSC-secreted HGF, inducing monocytes into a tolerogenic phenotype that then results in the modulation of both $CD8^+$ T cell responses as well as $CD4^+$ T cell responses, thus altering the cytokine milieu from a Th1 to a Th2 axis [43,44]. MSC-secreted HGF also contributes to the expansion of myeloid-derived suppressor cells (MDSCs) [45], which are a population of immature myeloid cells critical in providing an immunomodulatory environment for cancer growth, including suppressing effector T cell responses and expanding Tregs [46–48]. Interestingly, early reports studying the role of MSC-secreted HGF, which is also highly secreted by other stromal cells and by many cancers [49], yielded mixed results with regard to T cell interactions [50,51]. The demonstration of MET expression (the receptor for HGF) on myeloid cells but not T lymphocytes [44,45,52] could explain the inconsistent data with regard to immunomodulation of T cells, as well as highlighting the complex interplay that exists between leukocyte populations.

Along with DCs, macrophages are one of the two progenies of monocytes. The prototypical phagocyte, macrophages are found throughout the tissues of the body and are important as part of the initial defense of the immune system. These leukocytes can also recruit adaptive immune cells, such as T lymphocytes, through secretion of cytokines as well as present antigen, similar to DCs. Like T cells and DCs, it is now evident that macrophages can exist in a more proinflammatory phenotype, termed M1 macrophages, or in a more immunomodulatory phenotype, termed M2 macrophages [40]. The importance of the M2 phenotype is increasingly appreciated, especially in the context

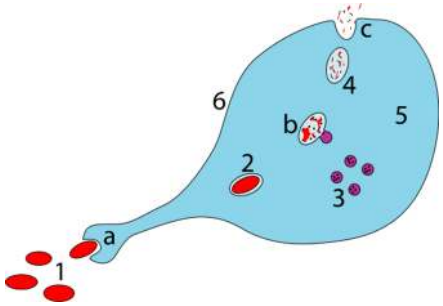



**Figure 33.4** Steps of a macrophage ingesting a pathogen. (a) Ingestion through phagocytosis, a phagosome is formed. (b) The fusion of lysosomes with the phagosome creates a phagolysosome; the pathogen is broken down by enzymes. (c) Waste material is expelled or assimilated (the latter not pictured). Parts: (1) pathogens; (2) phagosome; (3) lysosomes; (4) waste material; (5) cytoplasm; (6) cell membrane. *Source:* http://commons.wikimedia.org/wiki/File:Phagocytosis_ZP.svg#/media/File:Phagocytosis_ZP.svg.

of solid cancers, since tumor-associated macrophages contribute to tumor growth and immune evasion [53]. One of the earliest reports on MSC–macrophage interactions showed that $PGE_2$ secreted by MSCs modulates macrophages from a proinflammatory phenotype to a more immunomodulatory phenotype in a mouse model of sepsis [54]. Subsequent reports generally show that MSCs modulate macrophages into an immunomodulatory M2 phenotype through both cell–cell contact and paracrine mechanisms [55–57]. A recent report demonstrated that MSCs can inhibit activation in macrophages of the NOD-like receptor family pyrin domain containing 3 inflammasome by secretion of stanniocalcin in response to lipopolysaccharide-activated macrophages [58]. Macrophages can also modulate MSC phenotype. Using synovium from patients with osteoarthritis containing both M1 and M2 macrophages, the chondrogenic ability of MSCs can be altered by the M1 macrophage [59]. Furthermore, MSCs co-cultured with conditioned medium of proinflammatory macrophages acquire a proinflammatory phenotype, increasing IL-6 and CXCL10 secretion [60]. These reports collectively demonstrate that MSCs modulate diverse populations of myeloid and monocytic leukocytes. With increasing evidence demonstrating the important roles of these leukocytes in inflammation and cancer, it is anticipated that MSC interactions with these innate leukocyte populations will be further clarified.

Figure 33.4 shows a cartoon of the mechanism by which macrophages ingest pathogens.

## 33.4 Interactions with natural killer lymphocytes

NK cells are an important component of the innate immune system, targeting virus-infected cells and tumor cells. With few exceptions, NK cells comprise the bulk of innate leukocytes derived from the lymphoid progenitor. These innate lymphocytes are highly cytotoxic and secrete large amounts of proinflammatory cytokines, such as TNF-α and IFN-γ, recognizing and responding to MHC molecules signifying "self" versus "non-self", rather than specific antigens which T and B lymphocytes of the adaptive immune system recognize [61]. MSCs appear to be able to suppress the proliferation and cytokine production of NK cells, as well as modulate NK cytolytic functions [62–64]. While stimulated NK cells can efficiently lyse autologous and allogeneic MSCs, MSCs are still more resistant to NK cytolysis than other cell types [25,63,65,66], and this resistance to NK cytolysis can be further augmented by either stimulation of MSCs by IFN-γ and upregulation of surface expression of MHC class I molecules on MSCs [65] or by TLR3 stimulation [67]. The suppressive effects of MSCs on NK cells are detrimental with regard to tumor cytolysis [68,69] and result in protection of cancer cells in both *in vitro* and *in vivo* studies [70,71].

The inhibitory effects of MSCs on NK cells involve both cell–cell contact and secreted paracrine factors, including $PGE_2$, TGF-β, and IDO [63,64]. It has been found that constitutive expression of serine protease inhibitor 9 by MSCs was involved in MSC suppression of NK cytolysis [72]. In terms of cell–cell contact mechanisms, NK cells have a large repertoire of activating and inhibitory surface receptors, and the overall expression levels of all of these receptors determine the final phenotype of the NK cells: to be more cytotoxic or secretory [61]. MSCs suppress NK activity through interacting with a number of NK receptors, including downregulation of activating receptors NKp30, NKp46, NKp44, and most consistently NKG2D [64,69,73] as well as upregulation of the inhibitor receptor, KIR [74].

The nonclassical MHC class I molecule HLA-G, in both its secreted and surface isoforms, has a distinct role in MSC suppression of NK functions [74,75]. First identified in choriocarcinoma and migratory trophoblasts, HLA-G is thought to confer protective effects for the fetus from the maternal immune system, including directly suppressing maternal NK cytotoxicity [76]. HLA-G can exist in several forms, with the best characterized being one of the four complete transmembrane forms, HLA-G1, the predominant *in vivo* form (the others being HLA-G2, HLA-G3, and HLA-G4) and one of the three soluble truncated forms, HLA-G5 (the others being HLA-G6 and HLA-G7). Unlike most MHC class I molecules, HLA-G has very low polymorphism and its expression in the adult is highly restricted. However, in certain pathologic states, including cancer and inflammatory diseases, expression of sHLA-G has been observed [77]. The receptors for HLA-G include immunoglobulin-like transcript (ILT)-2, ILT-4, and CD94 and are found on a number of leukocytes, most prominently on NK cells [78]. Interestingly, embryonic and fetal MSCs can express HLA-G as a surface protein constitutively, and this ability has been speculated to be due to the earlier developmental stage of these cells [73,79]. Compared with adult bone marrow MSCs, fetal MSCs have a different expression profile of MHC molecules and responsiveness to IFN-γ stimulation [26]. Placental MSCs were

shown to preferentially upregulate surface HLA-G but not HLA-DR (an MHC class II molecule) after IFN-γ stimulation in contrast to adult bone marrow MSCs [74]. In embryonic and fetal MSCs, surface HLA-G expression appears to play a role in mediating modulation of NK responses [73,74]. There is still a role for HLA-G in adult bone marrow MSC suppression of NK cells, but it is in the form of sHLA-G rather than surface HLA-G [75]. The placenta is known to have unique immunomodulatory interactions with maternal uterine NK cells, which have a different effector profile than peripheral blood NK cells [80]. Thus, interactions of fetal MSCs with NK cells may differ from those of adult MSCs. Both fetal and adult gestational products are good sources of MSCs, and NK interactions may be quite varied depending on the developmental stage of the MSCs used. Mechanisms found for MSC immunomodulation of NK cells resemble those utilized by placental trophoblasts [81]. Research in this area of MSC-immune cell modulation will likely not only have clinical implications, but may also shed light on maternal–fetal immunodulation.

## 33.5 Interactions with neutrophils, other granulocytes, and mast cells

Neutrophils are the most abundant leukocyte in the human immune system [82]. A major type of granulocyte and an essential component of the innate immune system, these abundant but short-lived cells are the first leukocytes to arrive at sites of inflammation, especially if bacterial pathogens are involved. Along with the basophil granulocytes and eosinophil granulocytes, neutrophils are also known as polymorphonuclear cells due to their multilobed nucleus. Given the abundance and critical role of neutrophils in the acute inflammatory response, it is surprising that there are so few reports of MSC interactions with this leukocyte. In the few reports using co-culture of MSCs with freshly isolated human neutrophils, it appears that diverse sources of MSCs, including bone marrow, adipose tissue, and placenta, can suppress neutrophil apoptosis or prolong its viability [83,84] with mechanisms involving MSC-secreted IL-6 [85,86]. However, the effects of MSCs on functional and antibacterial activities of neutrophils, including phagocytosis and respiratory burst activity, appear to vary depending on the tissue source of MSCs utilized in the studies. Neutrophil migration and proinflammatory cytokine expression is enhanced after co-culture with salivary gland MSCs [87], and antibacterial activity, including phagocytosis, is enhanced after co-culture with either bone marrow or salivary gland MSCs [83,88] but not placental MSCs [85]. In murine models of bacteria-mediated lung injury, addition of MSCs reduced neutrophil influx and numbers, resulting in a significant decrease of inflammation and injury [89,90]. Neutrophils develop in the bone marrow from the common myeloid progenitor. However, only a few reports so far have begun to assess the niche/microenvironment aspect of MSC–neutrophil interactions [86,91]. Studies of the role of MSCs in the *in vivo* niche for neutrophil development can be anticipated to yield important insights into the physiological role of MSCs.

Reports of MSC interactions with other granulocytes (eosinophils and basophils) and with mast cells are scarce. These three types of innate immune system cells are prominent in allergic diseases, such as asthma. These leukocytes are mentioned in animal studies evaluating MSC treatment for asthma. It appears that MSC infusion in models of allergic airway inflammation generally results in decreased influx and degranulation of granulocytes and mast cells, leading to suppression of symptoms [92–94]. Decreased mast cell degranulation was also noted in a mouse model of contact dermatitis after treatment with MSCs isolated from the gingiva [95]. As shown by these few studies, the overall effect of MSCs on granulocytes and mast cells, and on allergic reactions in general, appears to be suppressive. Allergic responses are well known to be mediated by Th2 lymphocytes, and an important outcome of MSC immunomodulation is to induce Th1 lymphocytes into a Th2 fate [43,44]. Yet surprisingly, these *in vivo* studies of asthma models do not show exacerbation of symptoms after MSC treatment. The alleviation of Th2 allergic responses by MSCs in asthma may be due to the strong capacity of MSCs to modulate other aspects of inflammation in these allergic diseases, including increasing Treg numbers and the suppression of mast cell responses, known in asthma to be the most critical and dominant cell on lymphocyte/Th2 responses [96]. This could explain the numerous and generally efficacious reports of MSC application in animal models of asthma/allergic airway disease and in studies using asthma patient samples [97–101] (and see Chapters 39 and 53). A phase 1b study using intravenous infusions of human placenta MSCs in people with idiopathic pulmonary fibrosis was shown to be safe, demonstrating that MSCs, which are concentrated in the lungs immediately after intravenous infusion, do not appear to exacerbate underlying lung disease [102]. Clearly, it will be interesting for future studies to clarify the mechanisms by which MSCs modulate mast cell and granulocyte function for effective application in allergic diseases in general.

## 33.6 Interactions with complement

Unique to the innate immune system is the complement system, which is a noncellular component of the immune system. This system consists of proteins and glycoproteins mainly synthesized by the liver, with significant amounts produced by tissue macrophages, blood monocytes, and epithelial cells of the genitourinal tract and gastrointestinal tract. The functions of the complement system are to opsonize and enhance phagocytosis of antigens, to aid in chemotaxis of macrophages and neutrophils, to aid in cell lysis and membrane rupture of foreign cells, and to agglutinate pathogens. While studies on MSC–complement interactions are very scarce, findings so far demonstrate that MSCs inhibit complement activation [103,104]. MSCs can

be lysed by complement as well [105,106], and cryopreservation and increased passaging worsens complement-mediated elimination of MSCs, which would decrease therapeutic efficacy [107]. Given the varied interactions of MSCs with specific leukocyte populations, research on MSC–complement interactions is expected to be complex as well, and so reaching a consensus is premature at this time.

## References


1 R. J. Ulevitch. Therapeutics targeting the innate immune system. *Nat Rev Immunol* 2004;**4**:512–520.
2 I. Mellman and R. M. Steinman. Dendritic cells: specialized and regulated antigen processing machines. *Cell* 2001;**106**:255–258.
3 X. X. Jiang, Y. Zhang, B. Liu, *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**:4120–4126.
4 A. J. Nauta, A. B. Kruisselbrink, E. Lurvink, *et al.* Mesenchymal stem cells inhibit generation and function of both $CD34^+$-derived and monocyte-derived dendritic cells. *J Immunol* 2006;**177**:2080–2087.
5 A. Aldinucci, L. Rizzetto, L. Pieri, *et al.* Inhibition of immune synapse by altered dendritic cell actin distribution: a new pathway of mesenchymal stem cell immune regulation. *J Immunol* 2010;**185**:5102–5110.
6 Q. Wang, B. Sun, D. Wang, *et al.* Murine bone marrow mesenchymal stem cells cause mature dendritic cells to promote T-cell tolerance. *Scand J Immunol* 2008;**68**:607–615.
7 B. Zhang, R. Liu, D. Shi, *et al.* Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* 2009;**113**:46–57.
8 Y. Deng, S. Yi, G. Wang, *et al.* Umbilical cord-derived mesenchymal stem cells instruct dendritic cells to acquire tolerogenic phenotypes through the IL-6-mediated upregulation of SOCS1. *Stem Cells Dev* 2014;**23**:2080–2092.
9 F. M. Abomaray, M. A. Al Jumah, B. Kalionis, *et al.* Human chorionic villous mesenchymal stem cells modify the functions of human dendritic cells, and induce an anti-inflammatory phenotype in $CD1^+$ dendritic cells. *Stem Cell Rev* 2015;**11** (3):423–441.
10 F. R. Li, X. G. Wang, C. Y. Deng, *et al.* Immune modulation of cotransplantation mesenchymal stem cells with islet on T and dendritic cells. *Clin Exp Immunol* 2010;**161**:357–363.
11 F. de Sá Silva, R. N. Ramos, D. C. de Almeida, *et al.* Mesenchymal stem cells derived from human exfoliated deciduous teeth (SHEDs) induce immune modulatory profile in monocyte-derived dendritic cells. *PLoS ONE* 2014;**9**:e98050.
12 X. Liu, X. Qu, Y. Chen, *et al.* Mesenchymal stem/stromal cells induce the generation of novel IL-10-dependent regulatory dendritic cells by SOCS3 activation. *J Immunol* 2012;**189**:1182–1192.
13 L. Chen, W. Zhang, H. Yue, *et al.* Effects of human mesenchymal stem cells on the differentiation of dendritic cells from $CD34^+$ cells. *Stem Cells Dev* 2007;**16**:719–731.
14 F. Djouad, L. M. Charbonnier, C. Bouffi, *et al.* Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 2007;**25**:2025–2032.
15 H. Y. Lai, M. J. Yang, K. C. Wen, *et al.* Mesenchymal stem cells negatively regulate dendritic lineage commitment of umbilical-cord-blood-derived hematopoietic stem cells: an unappreciated mechanism as immunomodulators. *Tissue Eng Part A* 2010;**16**:2987–2997.
16 H. Li, Z. Guo, X. Jiang, *et al.* Mesenchymal stem cells alter migratory property of T and dendritic cells to delay the development of murine lethal acute graft-versus-host disease. *Stem Cells* 2008;**26**:2531–2541.
17 S. Beyth, Z. Borovsky, D. Mevorach, *et al.* Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;**105**:2214–2219.
18 Y. S. Choi, J. A. Jeong, and D. S. Lim. Mesenchymal stem cell-mediated immature dendritic cells induce regulatory T cell-based immunosuppressive effect. *Immunol Invest* 2012;**41**:214–229.
19 S. Chiesa, S. Morbelli, S. Morando, *et al.* Mesenchymal stem cells impair *in vivo* T-cell priming by dendritic cells. *Proc Natl Acad Sci U S A* 2011;**108**:17384–17389.
20 M. K. Majumdar, M. Keane-Moore, D. Buyaner, *et al.* Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* 2003;**10**:228–241.
21 W. K. Chan, A. S. Lau, J. C. Li, *et al.* MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-γ challenge. *Exp Hematol* 2008;**36**:1545–1555.
22 J. Stagg, S. Pommey, N. Eliopoulos, and J. Galipeau. Interferon-γ-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006;**107**:2570–2577.
23 R. Romieu-Mourez, M. Francois, M. N. Boivin, *et al.* Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. *J Immunol* 2009;**182**:7963–7973.
24 F. Morandi, L. Raffaghello, G. Bianchi, *et al.* Immunogenicity of human mesenchymal stem cells in HLA-class I-restricted T-cell responses against viral or tumor-associated antigens. *Stem Cells* 2008;**26**:1275–1287.
25 C. J. Chang, M. L. Yen, Y. C. Chen, *et al.* Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-γ. *Stem Cells* 2006;**24**:2466–2477.
26 C. Gotherström, O. Ringdén, C. Tammik, *et al.* Immunologic properties of human fetal mesenchymal stem cells. *Am J Obstet Gynecol* 2004;**190**:239–245.
27 O. Parolini, F. Alviano, G. P. Bagnara, *et al.* Concise review: isolation and characterization of cells from human term placenta: outcome of the first international Workshop on Placenta Derived Stem Cells. *Stem Cells* 2008;**26**:300–311.
28 F. Liotta, R. Angeli, L. Cosmi, *et al.* Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 2008;**26**:279–289.
29 M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, *et al.* Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 2007;**109**:1422–1432.
30 S. L. Tomchuck, K. J. Zwezdaryk, S. B. Coffelt, *et al.* Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells* 2008;**26**:99–107.
31 C. A. Dumitru, H. Hemeda, M. Jakob, *et al.* Stimulation of mesenchymal stromal cells (MSCs) via TLR3 reveals a novel mechanism of autocrine priming. *FASEB J* 2014;**28** (9):3856–3866.
32 S. H. Hwang, H. K. Cho, S. H. Park, *et al.* Toll like receptor 3 & 4 responses of human turbinate derived mesenchymal stem cells:

stimulation by double stranded RNA and lipopolysaccharide. *PLoS ONE* 2014;**9**:e101558.

33 T. V. Lanz, C. A. Opitz, P. P. Ho, *et al.* Mouse mesenchymal stem cells suppress antigen-specific TH cell immunity independent of indoleamine 2,3-dioxygenase 1 (IDO1). *Stem Cells Dev* 2010;**19**:657–668.

34 R. S. Waterman, S. L. Tomchuck, S. L. Henkle, and A. M. Betancourt. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* 2010;**5**:e10088.

35 X. Zhao, D. Liu, W. Gong, *et al.* The toll-like receptor 3 ligand, poly (I:C), improves immunosuppressive function and therapeutic effect of mesenchymal stem cells on sepsis via inhibiting MiR-143. *Stem Cells* 2014;**32**:521–533.

36 J. Lei, Z. Wang, D. Hui, *et al.* Ligation of TLR2 and TLR4 on murine bone marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity. *Cell Immunol* 2011;**271**:147–156.

37 G. Raicevic, M. Najar, B. Stamatopoulos, *et al.* The source of human mesenchymal stromal cells influences their TLR profile as well as their functional properties. *Cell Immunol* 2011;**270**:207–216.

38 S. Kern, H. Eichler, J. Stoeve, *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**:1294–1301.

39 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

40 S. Gordon and P. R. Taylor. Monocyte and macrophage heterogeneity. *Nat Rev Immunol* 2005;**5**:953–964.

41 M. E. Groh, B. Maitra, E. Szekely, and O. N. Koc. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol* 2005;**33**:928–934.

42 A. J. Cutler, V. Limbani, J. Girdlestone, and C. V. Navarrete. Umbilical cord-derived mesenchymal stromal cells modulate monocyte function to suppress T cell proliferation. *J Immunol* 2010;**185**:6617–6623.

43 I. Hof-Nahor, L. Leshansky, S. Shivtiel, *et al.* Human mesenchymal stem cells shift $CD8^+$ T cells towards a suppressive phenotype by inducing tolerogenic monocytes. *J Cell Sci* 2012;**125**:4640–4650.

44 P. M. Chen, K. J. Liu, P. J. Hsu, *et al.* Induction of immunomodulatory monocytes by human mesenchymal stem cell-derived hepatocyte growth factor through ERK1/2. *J Leukoc Biol* 2014;**96**:295–303.

45 B. L. Yen, M. L. Yen, P. J. Hsu, *et al.* Multipotent mesenchymal stromal cells mediate the expansion of myeloid-derived suppressor cells through the HGF/c-met axis and STAT3. *Stem Cell Rep* 2013;**1**:139–151.

46 S. Ostrand-Rosenberg and P. Sinha. Myeloid-derived suppressor cells: linking inflammation and cancer. *J Immunol* 2009;**182**:4499–4506.

47 K. Kim, A. D. Skora, Z. Li, *et al.* Eradication of metastatic mouse cancers resistant to immune checkpoint blockade by suppression of myeloid-derived cells. *Proc Natl Acad Sci U S A* 2014;**111**:11774–11779.

48 A. S. Dugast, T. Haudebourg, F. Coulon, *et al.* Myeloid-derived suppressor cells accumulate in kidney allograft tolerance and specifically suppress effector T cell expansion. *J Immunol* 2008;**180**:7898–7906.

49 M. M. Mueller and N. E. Fusenig. Friends or foes – bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* 2004;**4**:839–849.

50 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.

51 K. Le Blanc, L. Tammik, B. Sundberg, *et al.* Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003;**57**:11–20.

52 M. Beilmann, M. Odenthal, W. Jung, *et al.* Neoexpression of the c-met/hepatocyte growth factor-scatter factor receptor gene in activated monocytes. *Blood* 1997;**90**:4450–4458.

53 P. Allavena, A. Sica, C. Garlanda, and A. Mantovani. The yin-yang of tumor-associated macrophages in neoplastic progression and immune surveillance. *Immunol Rev* 2008;**222**:155–161.

54 K. Nemeth, A. Leelahavanichkul, P. S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

55 M. Pietila, S. Lehtonen, E. Tuovinen, *et al.* CD200 positive human mesenchymal stem cells suppress TNF-α secretion from CD200 receptor positive macrophage-like cells. *PLoS ONE* 2012;**7**:e31671.

56 M. H. Abumaree, M. A. Al Jumah, B. Kalionis, *et al.* Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages. *Stem Cell Rev* 2013;**9**:620–641.

57 A. F. Wise, T. M. Williams, M. B. Kiewiet, *et al.* Human mesenchymal stem cells alter macrophage phenotype and promote regeneration via homing to the kidney following ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2014;**306**:F1222–F1235.

58 J. Y. Oh, J. H. Ko, H. J. Lee, *et al.* Mesenchymal stem/stromal cells inhibit the NLRP3 inflammasome by decreasing mitochondrial reactive oxygen species. *Stem Cells* 2014;**32**:1553–1563.

59 N. Fahy, M. L. de Vries-van Melle, J. Lehmann, *et al.* Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. *Osteoarthritis Cartilage* 2014;**22**:1167–1175.

60 K. Anton, D. Banerjee, and J. Glod. Macrophage-associated mesenchymal stem cells assume an activated, migratory, pro-inflammatory phenotype with increased IL-6 and CXCL10 secretion. *PLoS ONE* 2012;**7**:e35036.

61 M. A. Caligiuri. Human natural killer cells. *Blood* 2008;**112**:461–469.

62 S. Aggarwal and M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.

63 P. A. Sotiropoulou, S. A. Perez, A. D. Gritzapis, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**:74–85.

64 G. M. Spaggiari, A. Capobianco, H. Abdelrazik, *et al.* Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008;**111**:1327–1333.

65 G. M. Spaggiari, A. Capobianco, S. Becchetti, *et al.* Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK

cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;**107**:1484–1490.

66 C. Menard, L. Pacelli, G. Bassi, *et al.* Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: standardization of immune quality controls. *Stem Cells Dev* 2013;**22**:1789–1801.

67 M. Giuliani, A. Bennaceur-Griscelli, A. Nanbakhsh, *et al.* TLR ligands stimulation protects MSC from NK killing. *Stem Cells* 2014;**32**:290–300.

68 Y. Li, Y. H. Qu, Y. F. Wu, *et al.* Bone marrow mesenchymal stem cells reduce the antitumor activity of cytokine-induced killer/natural killer cells in K562 NOD/SCID mice. *Ann Hematol* 2011;**90**:873–885.

69 Z. G. Zhao, Z. Cao, W. Xu, *et al.* Immune protection function of multipotent mesenchymal stromal cells: role of transforming growth factor-β1. *Cancer Invest* 2012;**30**:646–656.

70 S. A. Patel, J. R. Meyer, S. J. Greco, *et al.* Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-β. *J Immunol* 2010;**184**:5885–5894.

71 B. Ljujic, M. Milovanovic, V. Volarevic, *et al.* Human mesenchymal stem cells creating an immunosuppressive environment and promote breast cancer in mice. *Sci Rep* 2013;**3**:2298.

72 N. El Haddad, R. Moore, D. Heathcote, *et al.* The novel role of SERPINB9 in cytotoxic protection of human mesenchymal stem cells. *J Immunol* 2011;**187**:2252–2260.

73 B. L. Yen, C. J. Chang, K. J. Liu, *et al.* Human embryonic stem cell-derived mesenchymal progenitors possess strong immunosuppressive effects toward natural killer cells as well as T lymphocytes. *Stem Cells* 2009;**27**:451–456.

74 K. J. Liu, C. J. Wang, C. J. Chang, *et al.* Surface expression of HLA-G is involved in mediating immunomodulatory effects of placenta-derived multipotent cells (PDMCs) towards natural killer lymphocytes. *Cell Transplant* 2011;**10–11**:1721–1730.

75 Z. Selmani, A. Naji, I. Zidi, *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**:212–222.

76 N. Rouas-Freiss, R. M. Goncalves, C. Menier, *et al.* Direct evidence to support the role of HLA-G in protecting the fetus from maternal uterine natural killer cytolysis. *Proc Natl Acad Sci U S A* 1997;**94**:11520–11525.

77 E. D. Carosella, B. Favier, N. Rouas-Freiss, *et al.* Beyond the increasing complexity of the immunomodulatory HLA-G molecule. *Blood* 2008;**111**:4862–4870.

78 J. LeMaoult, K. Zafaranloo, C. Le Danff, and E. D. Carosella. HLA-G up-regulates ILT2, ILT3, ILT4, and KIR2DL4 in antigen presenting cells, NK cells, and T cells. *FASEB J* 2005;**19**:662–664.

79 M. L. Weiss, C. Anderson, S. Medicetty, *et al.* Immune properties of human umbilical cord Wharton's jelly-derived cells. *Stem Cells* 2008;**26**:2865–2874.

80 A. Moffett-King. Natural killer cells and pregnancy. *Nat Rev Immunol* 2002;**2**:656–663.

81 A. Nasef, N. Mathieu, A. Chapel, *et al.* Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation* 2007;**84**:231–237.

82 C. Nathan. Neutrophils and immunity: challenges and opportunities. *Nat Rev Immunol* 2006;**6**:173–182.

83 M. A. Cassatella, F. Mosna, A. Micheletti, *et al.* Toll-like receptor-3-activated human mesenchymal stromal cells significantly prolong the survival and function of neutrophils. *Stem Cells* 2011;**29**:1001–1011.

84 Y. S. Park, G. W. Lim, K. A. Cho, *et al.* Improved viability and activity of neutrophils differentiated from HL-60 cells by co-culture with adipose tissue-derived mesenchymal stem cells. *Biochem Biophys Res Commun* 2012;**423**:19–25.

85 C. P. Chen, Y. Y. Chen, J. P. Huang, and Y. H. Wu. The effect of conditioned medium derived from human placental multipotent mesenchymal stromal cells on neutrophils: possible implications for placental infection. *Mol Hum Reprod* 2014;**20**:1117–1125.

86 L. Raffaghello, G. Bianchi, M. Bertolotto, *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 2008;**26**:151–162.

87 S. Brandau, M. Jakob, H. Hemeda, *et al.* Tissue-resident mesenchymal stem cells attract peripheral blood neutrophils and enhance their inflammatory activity in response to microbial challenge. *J Leukoc Biol* 2010;**88**:1005–1015.

88 S. Brandau, M. Jakob, K. Bruderek, *et al.* Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes. *PLoS ONE* 2014;**9**:e106903.

89 J. W. Lee, X. Fang, N. Gupta, *et al.* Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the *ex vivo* perfused human lung. *Proc Natl Acad Sci U S A* 2009;**106**:16357–16362.

90 Y. G. Zhu, X. M. Feng, J. Abbott, *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;**32**:116–125.

91 S. C. Hsu, L. T. Wang, C. L. Yao, *et al.* Mesenchymal stem cells promote neutrophil activation by inducing IL-17 production in $CD4^+$ $CD45RO^+$ T cells. *Immunobiology* 2013;**218**:90–95.

92 H. F. Ou-Yang, Y. Huang, X. B. Hu, and C. G. Wu. Suppression of allergic airway inflammation in a mouse model of asthma by exogenous mesenchymal stem cells. *Exp Biol Med (Maywood)* 2011;**236**:1461–1467.

93 Y. Q. Sun, M. X. Deng, J. He, *et al.* Human pluripotent stem cell-derived mesenchymal stem cells prevent allergic airway inflammation in mice. *Stem Cells* 2012;**30**:2692–2699.

94 S. H. Lee, A. S. Jang, J. H. Kwon, *et al.* Mesenchymal stem cell transfer suppresses airway remodeling in a toluene diisocyanate-induced murine asthma model. *Allergy Asthma Immunol Res* 2011;**3**:205–211.

95 W. R. Su, Q. Z. Zhang, S. H. Shi, *et al.* Human gingiva-derived mesenchymal stromal cells attenuate contact hypersensitivity via prostaglandin E2-dependent mechanisms. *Stem Cells* 2011;**29**:1849–1860.

96 W. W. Busse and R. F. Lemanske, Jr. Asthma. *N Engl J Med* 2001;**344**:350–362.

97 T. L. Bonfield, M. Koloze, D. P. Lennon, *et al.* Human mesenchymal stem cells suppress chronic airway inflammation in the murine ovalbumin asthma model. *Am J Physiol Lung Cell Mol Physiol* 2010;**299**:L760–L770.

98 S. Kapoor, S. A. Patel, S. Kartan, *et al.* Tolerance-like mediated suppression by mesenchymal stem cells in patients with dust mite

allergy-induced asthma. *J Allergy Clin Immunol* 2012;**129**: 1094–1101.

99 L. J. Mathias, S. M. Khong, L. Spyroglou, *et al.* Alveolar macrophages are critical for the inhibition of allergic asthma by mesenchymal stromal cells. *J Immunol* 2013;**191**:5914–5924.

100 P. Gao, Y. Zhou, L. Xian, *et al.* Functional effects of TGF-β1 on mesenchymal stem cell mobilization in cockroach allergen-induced asthma. *J Immunol* 2014;**192**:4560–4570.

101 J. E. Trzil, I. Masseau, T. L. Webb, *et al.* Long term evaluation of mesenchymal stem cell therapy in a feline model of chronic allergic asthma. *Clin Exp Allergy* 2014;**44** (12):1546–1557.

102 D. C. Chambers, D. Enever, N. Ilic, *et al.* A phase 1b study of placenta-derived mesenchymal stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2014;**19**:1013–1018.

103 Z. Tu, Q. Li, H. Bu, and F. Lin. Mesenchymal stem cells inhibit complement activation by secreting factor H. *Stem Cells Dev* 2010;**19**:1803–1809.

104 G. Moll, R. Jitschin, L. von Bahr, *et al.* Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS ONE* 2011;**6**:e21703.

105 Y. Li and F. Lin. Mesenchymal stem cells are injured by complement after their contact with serum. *Blood* 2012;**120**:3436–3443.

106 M. A. Soland, M. Bego, E. Colletti, *et al.* Mesenchymal stem cells engineered to inhibit complement-mediated damage. *PLoS ONE* 2013;**8**:e60461.

107 G. Moll, J. J. Alm, L. C. Davies, *et al.* Do cryopreserved mesenchymal stromal cells display impaired immunomodulatory and therapeutic properties? *Stem Cells* 2014;**32**:2430–2342.

CHAPTER 34

# The modulatory effects of mesenchymal stromal cells on the adaptive immune system

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## 34.1 Introduction to the adaptive immune system

The adaptive arm of the immune system is comprised of T and B lymphocytes. In contrast to the evolutionarily older innate immune system, the adaptive immune system is highly specific and able to confer immunological memory, which allows for an enhanced response when the same pathogen is encountered again. This is achieved through the ability of T and B lymphocytes to recognize an enormous array of highly specific antigens, another advantage compared with the innate immune response. However, the adaptive immune response is slower than innate leukocyte responses. These differences between the two arms of the immune system are clearly complementary to each other, and complete the powerful armamentarium that humans and other mammals have against pathogens and disease. A schema of the immunomodulatory mechanisms of mesenchymal stromal cells (MSCs) on lymphocytes of the adaptive immune system is shown in Figure 34.1.

## 34.2 Interactions with T lymphocytes

T lymphocytes are responsible for the cell-mediated responses of the adaptive immune system. The importance of this class of leukocytes is clearly demonstrated by the wide range and types of diseases due to abnormalities in this area, including autoimmune diseases, allergies, and some immunodeficiencies. Moreover, recipient and donor cytotoxic $CD8^+$ T lymphocytes play a major role in the rejection of transplanted organs and in the mediation of graft-versus-host-disease respectively. On the other hand, immunomodulatory T lymphocytes have been found in solid tumors and contribute to the immunosuppressive niche. It is no surprise, then, that of all the leukocyte subpopulations, MSC interactions with T lymphocytes are the best studied to date. Current understanding of MSC interactions with T lymphocytes demonstrates that MSCs can strongly suppress effector T cell functions [1,2]. The evidence is best demonstrated for adult bone-marrow-derived MSCs (BM-MSCs), but fetal source and adipose-tissue MSCs also appear to harbor similar properties [3,4]. Concomitantly, and a nearly universal finding in MSC–T-cell studies, is the resulting decrease in secretion of proinflammatory cytokines, most consistently being tumor necrosis factor-α and IFN-γ, which are also considered to be part of the Th1 response [5–7]. Most of these studies examined T effector cells in general, encompassing both $CD4^+$ helper and $CD8^+$ cytotoxic T cells, but a few reports have focused on $CD8^+$ cytotoxic T cells and found that MSCs efficiently suppress the proliferation and cytolytic potential of these T cells as well [6,8–15]. MSC suppression of T lymphocyte proliferation has been demonstrated to be due to cell cycle arrest of activated T cells in the in the $G_0/G_1$ phase [16], and not involving apoptosis [15,17,18]. However, a few reports have shown that MSCs preferentially induce apoptosis of activated T cell subpopulations [19], and a more recent study in mouse models

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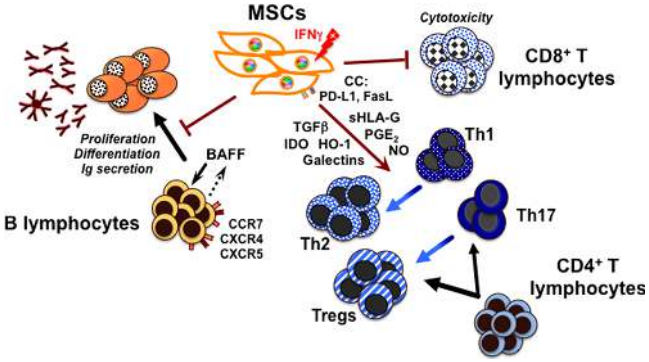



**Figure 34.1** Immunomodulatory mechanisms of MSCs on lymphocytes of the adaptive immune system. IFN-γ:, interferon-γ; Ig: immunoglobulin; BAFF: B-cell activating factor (dotted arrow indicates secretion by B lymphocytes); CC: cell contact; PD-L1: programmed death-ligand 1; FasL: Fas ligand; TGF-β: transforming growth factor-β; IDO: indoleamine 2,3-dioxyenase; HO-1: heme oxygenase-1; $PGE_2$: prostaglandin $E_2$; sHLA-G: soluble human leukocyte antigen-G; NO: nitric oxide; Th: T helper; Tregs: regulatory T cells.

demonstrated that systemic infusion of allogeneic murine BM-MSCs induced transient T cell apoptosis via FasL. The authors found that the presence of FasL on MSCs was critical to its therapeutic efficacy in mouse disease models of systemic sclerosis (scleroderma) and colitis [20]. Downregulation of the Fas receptor/FasL axis on T cells by MSCs has also been shown to prolong the survival of unstimulated T cells in a quiescent state [21]. Interestingly, a recent study found that telomerase can induce upregulation of FasL in MSCs, which affects immunomodulatory properties [22]. Despite mechanistic differences found for the role of the Fas/FasL axis in MSC-mediated suppression of T lymphocytes, the overall effect appears to be one of immunomodulation and T cell quiescence/nonproliferation.

The interactions of MSCs with T cells can modulate the fate of these lymphocytes, and reports are most consistent in showing MSC modulation of $CD4^+$ T cells toward Tregs [23,24]. Tregs, noted as being $CD4^+$ $CD25^{high}$ cells, are a population of immunomodulatory lymphocytes that play an important role in the induction of peripheral tolerance and the inhibition of proinflammatory immune responses [25]. Tregs are known to secrete interleukin (IL)-10 and TGF-β (two immunomodulatory factors), and lack of this $CD4^+$ population results in the development of severe, multiple organ autoimmune diseases in a number of mouse models [26,27]. A critical transcription factor involved in the differentiation of $CD4^+$ naive T cells to Tregs is $Foxp3^+$, and most studies show that co-culture of MSCs with peripheral blood mononuclear cells or with $CD4^+$ cells can induce the expansion of functional $Foxp3^+$ $CD4^+$ $CD25^{high}$ Tregs [18,28–34]. MSCs have also been shown in a few studies to induce a subpopulation of Tregs, the type 1 Tregs (Tr1), which secrete IFN-γ and IL-10 [35,36]. In studies with animal disease models of autoimmune disease or organ transplantation, the induction of Tregs by MSCs is clearly involved in mediating therapeutic responses [33,37–39]. Thus, both *in vitro* and *in vivo* data support the capacity of MSCs to induce Tregs and the critical role of this effect in ameliorating disease.

One population of $CD4^+$ T lymphocytes that has become prominent recently is the IL-17A-secreting T cell population [40]. Also known as Th17 cells, this T helper cell subpopulation is important in mediating host responses toward microbial infections as well as participating in the pathogenesis of many autoimmune and chronic inflammatory diseases that had long been believed to be caused by Th1 cells [41]. Th17 lineages are also linked to Tregs, and it is currently believed that one lineage is chosen over another to maintain immune homeostasis [42]. With the majority of studies demonstrating the induction of Tregs by MSCs, it should come as no surprise that many reports show that MSCs suppress the formation of Th17 cells both in *in vitro* studies and in *in vivo* disease models [23,43–47]. In fact, a study using a *Helicobacter pylori* model of gastric cancer in mice found that MSC modulation of $CD4^+$ cells away from Th17 cells and toward Tregs may create a permissive environment for the development of gastric cancer [48]. However, some studies have found that MSCs actually enhance Th17 responses [49–51]. This may be dependent on the specific molecules expressed by MSCs [52]. Given the importance of the Th17 population in the pathogenesis of many diseases, further investigation is warranted to elucidate the complexities of MSC interactions with this critical $CD4^+$ subpopulation.

Numerous studies have attempted to delineate whether MSC–T-cell interactions are mediated through cell–cell contact or by secretion of factors. Cell contact appears to play a partial role [30,53,54], but specific ligand–receptor interaction has been surprisingly difficult to discern with the exception of the programmed cell death (PD) PD-1/PD-L1 pathway [47,55,56]. This may be due to the overwhelming effects of MSC paracrine factors on T cell function [3,57]. One of the first paracrine molecules found to mediate MSC immunomodulatory effects on T cells was TGF-β, itself an immunomodulatory molecule [8,17,18,30]. MSCs can also induce leukocytes to express TGF-β, further enhancing the overall immunosuppressive environment [15,56,58,59]. Many studies have also demonstrated the involvement of other MSC-secreted factors, including IDO [1,8,19,36,60,61], $PGE_2$ [8,23,30,62], sHLA-G5 [28,63], HO-1 [35,64], NO [65–67], and various galectins [68–71]. MSC secretion of these many immunomodulatory molecules may be constitutive, but clearly is enhanced by the proinflammatory cytokine IFN-γ [1,13,18,60,69]. This may be one explanation for the strong capacity of MSCs to be therapeutic in highly inflammatory environments, such as graft-versus-host-disease [72].

Despite the overwhelming number of studies showing MSC downmodulation of T cell effector functions, there are some reports showing enhancement of T cell responses [73]. One important reason for this contrasting study may be due to differences in data obtained from *in vitro* versus *in vivo* settings [74,75]. Another point to consider is the subtle phenotypic

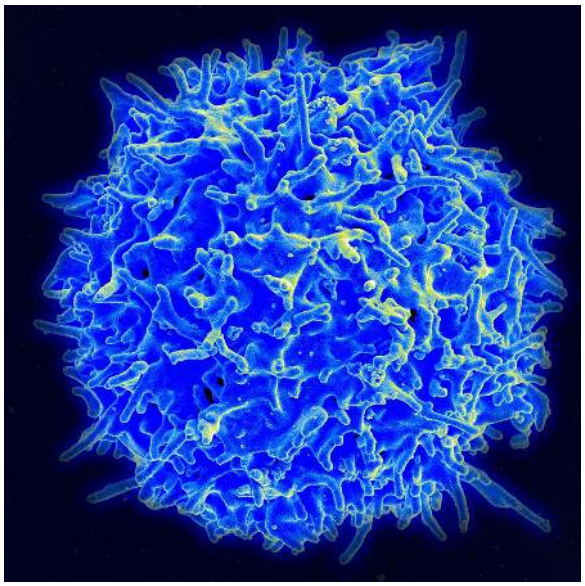


**Figure 34.2** Scanning electron micrograph of a human T cell of a healthy donor. *Source*: Healthy Human T Cell by NIAID/NIH.

variations due to the many methods available for isolation of MSCs [76]. An important caveat is that nearly all reported *in vitro* studies have used healthy donor T cells. In studies using patient T cells, decreased responsiveness can be observed [77,78]. This is a critical point to keep in mind and could be one reason why clinical trials using MSCs for immune-related diseases have not been as positive as might have been presumed [79]. Given the importance of the T lymphocyte in immune diseases and related conditions, it is crucial to establish a clear mechanistic understanding of MSC–T-cell interactions to obtain efficaceous clinical applications.

A scanning electomicraph of a normal human T cell is shown in Figure 34.2.

## 34.3 Interactions with B lymphocytes

B lymphocytes are responsible for the humoral response of the adaptive immune system. In contrast to T lymphocytes, which mediate cell-directed effector functions, B cells secrete proteins known as antibodies with high specificity for particular antigens. These lymphocytes can also be APCs, able to recognize cognate antigens in their native form, unlike T cells, which recognize antigens only after processing by an APC. Abnormalities in B cells result in a complete or partial absence of a particular class of antibodies, one or more immunoglobulin isotypes, with symptoms being recurrent infections with specific microorganisms.

One of the first reports on MSC–B-cell interactions demonstrated that MSCs, through paracrine factors, reduced B cell production of IgM, IgG, and IgA and affected B cell chemotaxis by downregulation of CXCR4, CXCR5, and CCR7 on B cells [80]. However, one report found that MSCs increased B cell production of IgG [81]. More consistently, most studies have shown that MSCs inhibit B cell differentiation, delaying maturation [80]. B cell proliferation was inhibited by MSCs through cell cycle arrest at the $G_0/G_1$ phase of the cell cycle and not through the induction of apoptosis [80]. MSC inhibition of B cell proliferation, differentiation, and antibody secretion has also been reported to require interactions with T cells first, involving both cell contact and secreted factors, as well as requiring both $CD4^+$ and $CD8^+$ T cells [82]. As with T cells, IFN-γ can also enhance MSC suppression of B cell function [83].

There are some convincing *in vivo* murine and human patient data showing suppressive effects of MSCs on B cell functions, and this involved decreasing B cell expression of BAFF, a potent B cell activator, by MSCs in a murine model of lupus nephritis [84] and in patients with chronic graft-versus-host disease who responded to the administration of MSCs [85].

B cells develop in the bone marrow and, as such, should have many interactions with BM-MSCs. It is therefore surprising that the number of studies on MSC–B-cell interactions lags far behind that of interactions with T cells. This may be due to the fact that pathologies in T cell biology dominate clinical disease entities. Moreover, developmental interactions may not be captured with *in vitro* studies (the predominant type of MSC–immune cell reports) and likely require *in vivo* modeling or complex *in vitro* systems [86,87]. A study using a genetically manipulated mouse model demonstrated that CCAAT/enhancer-binding protein β expression by BM-MSCs is essential in early B cell development [88]. It is anticipated that studies using such genetic *in vivo* models or complex *in vitro* models will yield further insights into MSC–B-lymphocyte niche interactions.

## References

1 M. Krampera, L. Cosmi, R. Angeli, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stromal cells. *Stem Cells* 2006;**24**:386–398.
2 M. Krampera, S. Glennie, J. Dyson, *et al.* Bone marrow mesenchymal stromal cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;**101**:3722–3729.
3 P. M. Chen, M. L. Yen, K. J. Liu, *et al.* Immunomodulatory properties of human adult and fetal multipotent mesenchymal stromal cells. *J Biomed Sci* 2011;**18**:49.
4 K. A. Keyser, K. E. Beagles, and H. P. Kiem, Comparison of mesenchymal stromal cells from different tissues to suppress T-cell activation. *Cell Transplant* 2007;**16**:555–562.
5 E. Zappia, S. Casazza, E. Pedemonte, *et al.* Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis inducing T-cell anergy. *Blood* 2005;**106**:1755–1761.
6 I. Rasmusson, M. Uhlin, K. Le Blanc, and V. Levitsky. Mesenchymal stromal cells fail to trigger effector functions of cytotoxic T lymphocytes. *J Leukoc Biol* 2007;**82**:887–893.
7 A. Dorronsoro, I. Ferrin, J. M. Salcedo, *et al.* Human mesenchymal stromal cells modulate T-cell responses through TNF-α-mediated activation of NF-κB. *Eur J Immunol* 2014;**44**:480–488.

8 M. Li, X. Sun, X. Kuang, *et al*. Mesenchymal stromal cells suppress $CD8^+$ T cell-mediated activation by suppressing NKG2D receptor expression and secretion of PGE2, IDO and TGF-β. *Clin Exp Immunol* 2014;**178** (3):516–524.

9 I. Hof-Nahor, L. Leshansky, S. Shivtiel, *et al*. Human mesenchymal stromal cells shift $CD8^+$ T cells towards a suppressive phenotype by inducing tolerogenic monocytes. *J Cell Sci* 2012;**125**:4640–4650.

10 A. U. Engela, C. C. Baan, N. H. Litjens, *et al*. Mesenchymal stromal cells control alloreactive $CD8^+$ $CD28^-$ T cells. *Clin Exp Immunol* 2013;**174**:449–458.

11 M. N. Zhou, Z. Q. Zhang, J. L. Wu, *et al*. Dermal mesenchymal stromal cells (DMSCs) inhibit skin-homing $CD8^+$ T cell activity, a determining factor of vitiligo patients' autologous melanocytes transplantation efficiency. *PLoS ONE* 2013;**8**:e60254.

12 G. Malcherek, N. Jin, A. G. Huckelhoven, *et al*. Mesenchymal stromal cells inhibit proliferation of virus-specific $CD8^+$ T cells. *Leukemia* 2014;**28** (12):2388–2394.

13 D. Wang, X. Feng, L. Lu, *et al*. A CD8T cell/indoleamine 2,3-dioxygenase axis is required for mesenchymal stem cell suppression of human systemic lupus erythematosus. *Arthritis Rheumatol* 2014;**66**:2234–2245.

14 I. Rasmusson, O. Ringdén, B. Sundberg, and K. Le Blanc. Mesenchymal stromal cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 2003;**76**:1208–1213.

15 W. T. Tse, J. D. Pendleton, W. M. Beyer, *et al*. Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003;**75**:389–397.

16 S. Glennie, I. Soeiro, P. J. Dyson, *et al*. Bone marrow mesenchymal stromal cells induce division arrest anergy of activated T cells. *Blood* 2005;**105**:2821–2827.

17 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.

18 C. J. Chang, M. L. Yen, Y. C. Chen, *et al*. Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-γ. *Stem Cells* 2006;**24**:2466–2477.

19 J. Plumas, L. Chaperot, M. J. Richard, *et al*. Mesenchymal stromal cells induce apoptosis of activated T cells. *Leukemia* 2005;**19**: 1597–1604.

20 K. Akiyama, C. Chen, D. Wang, *et al*. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012;**10**:544–555.

21 F. Benvenuto, S. Ferrari, E. Gerdoni, *et al*. Human mesenchymal stromal cells promote survival of T cells in a quiescent state. *Stem Cells* 2007;**25**:1753–1760.

22 C. Chen, K. Akiyama, T. Yamaza, *et al*. Telomerase governs immunomodulatory properties of mesenchymal stromal cells by regulating FAS ligand expression. *EMBO Mol Med* 2014;**6**:322–334.

23 S. Aggarwal and M. F. Pittenger. Human mesenchymal stromal cells modulate allogeneic immune cell responses. *Blood* 2005;**105**: 1815–1822.

24 R. Maccario, M. Podestà, A. Moretta, *et al*. Interaction of human mesenchymal stromal cells with cells involved in alloantigen-specific immune response favors the differentiation of $CD4^+$ T-cell subsets expressing a regulatory/suppressive phenotype. *Haematologica* 2005;**90**:516–525.

25 J. L. Riley, C. H. June, and B. R. Blazar. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* 2009;**30**:656–665.

26 E. M. Shevach. Mechanisms of $Foxp3^+$ T regulatory cell-mediated suppression. *Immunity* 2009;**30**:636–645.

27 M. Itoh, T. Takahashi, N. Sakaguchi, *et al*. Thymus and autoimmunity: production of $CD25^+CD4^+$ naturally anergic and suppressive T cells as a key function of the thymus in maintaining immunologic self-tolerance. *J Immunol* 1999;**162**:5317–5326.

28 Z. Selmani, A. Naji, I. Zidi, *et al*. Human leukocyte antigen-G5 secretion by human mesenchymal stromal cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**: 212–222.

29 M. Di Ianni, B. Del Papa, M. De Ioanni, *et al*. Mesenchymal cells recruit and regulate T regulatory cells. *Exp Hematol* 2008;**36**: 309–318.

30 K. English, J. M. Ryan, L. Tobin, *et al*. Cell contact, prostaglandin $E_2$ and transforming growth factor β1 play non-redundant roles in human mesenchymal stem cell induction of $CD4^+CD25^{High}$ forkhead box $P3^+$ regulatory T cells. *Clin Exp Immunol* 2009;**156**: 149–160.

31 T. Takahashi, A. Tibell, K. Ljung, *et al*. Multipotent mesenchymal stromal cells synergize with costimulation blockade in the inhibition of immune responses and the induction of $Foxp3^+$ regulatory T cells. *Stem Cells Transl Med* 2014;**3** (12):1484–1494.

32 R. S. Thakur, S. Tousif, V. Awasthi, *et al*. Mesenchymal stromal cells play an important role in host protective immune responses against malaria by modulating regulatory T cells. *Eur J Immunol* 2013;**43**:2070–2077.

33 P. Luz-Crawford, M. Kurte, J. Bravo-Alegria, *et al*. Mesenchymal stromal cells generate a $CD4^+CD25^+Foxp3^+$ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem Cell Res Ther* 2013;**4**:65.

34 B. Del Papa, P. Sportoletti, D. Cecchini, *et al*. Notch1 modulates mesenchymal stromal cells mediated regulatory T-cell induction. *Eur J Immunol* 2013;**43**:182–187.

35 D. Mougiakakos, R. Jitschin, C. C. Johansson, *et al*. The impact of inflammatory licensing on heme oxygenase-1-mediated induction of regulatory T cells by human mesenchymal stromal cells. *Blood* 2011;**117**:4826–4835.

36 W. T. Hsu, C. H. Lin, B. L. Chiang, *et al*. Prostaglandin E2 potentiates mesenchymal stem cell-induced $IL\text{-}10^+IFN\text{-}\gamma^+CD4^+$ regulatory T cells to control transplant arteriosclerosis. *J Immunol* 2013;**190**: 2372–2380.

37 Y. Wang, A. Zhang, Z. Ye, H. Xie, and S. Zheng. Bone marrow-derived mesenchymal stromal cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion. *Transplant Proc* 2009;**41**:4352–4356.

38 W. Ge, J. Jiang, J. Arp, *et al*. Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stromal cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* 2010;**90**:1312–1320.

39 J. H. Lee, E. J. Jeon, N. Kim, *et al*. The synergistic immunoregulatory effects of culture-expanded mesenchymal stromal cells and $CD4^+25^+Foxp3^+$ regulatory T cells on skin allograft rejection. *PLoS ONE* 2013;**8**:e70968.

40 C. Dong. IL-23/IL-17 biology and therapeutic considerations. *J Immunotoxicol* 2008;**5**:43–46.

41 P. Miossec and J. K. Kolls. Targeting IL-17 and TH17 cells in chronic inflammation. *Nat Rev Drug Discov* 2012;**11**:763–776.

42 C. T. Weaver and R. D. Hatton. Interplay between the TH17 and TReg cell lineages: a (co-)evolutionary perspective. *Nat Rev Immunol* 2009;**9**:883–889.

43 L. Bai, D. P. Lennon, V. Eaton, *et al*. Human bone marrow-derived mesenchymal stromal cells induce Th2-polarized immune response and promote endogenous repair in animal models of multiple sclerosis. *Glia* 2009;**57**:1192–1203.

44 M. Rafei, P. M. Campeau, A. Aguilar-Mahecha, *et al*. Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 2009;**182**:5994–6002.

45 S. Ghannam, J. Pene, G. Moquet-Torcy, *et al*. Mesenchymal stromal cells inhibit human Th17 cell differentiation and function and induce a T regulatory cell phenotype. *J Immunol* 2010;**185**:302–312.

46 M. Chen, W. Su, X. Lin, *et al*. Adoptive transfer of human gingiva-derived mesenchymal stromal cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation. *Arthritis Rheum* 2013;**65**: 1181–1193.

47 P. Luz-Crawford, D. Noël, X. Fernandez, *et al*. Mesenchymal stromal cells repress Th17 molecular program through the PD-1 pathway. *PLoS ONE* 2012;**7**:e45272.

48 R. Lin, H. Ma, Z. Ding, *et al*. Bone marrow-derived mesenchymal stromal cells favor the immunosuppressive T cells skewing in a *Helicobacter pylori* model of gastric cancer. *Stem Cells Dev* 2013;**22**:2836–2848.

49 P. J. Darlington, M. N. Boivin, C. Renoux, *et al*. Reciprocal Th1 and Th17 regulation by mesenchymal stromal cells: implication for multiple sclerosis. *Ann Neurol* 2010;**68**:540–545.

50 G. H. Tso, H. K. Law, W. Tu, *et al*. Phagocytosis of apoptotic cells modulates mesenchymal stromal cells osteogenic differentiation to enhance IL-17 and RANKL expression on $CD4^+$ T cells. *Stem Cells* 2010;**28**:939–954.

51 S. C. Hsu, L. T. Wang, C. L. Yao, *et al*. Mesenchymal stromal cells promote neutrophil activation by inducing IL-17 production in $CD4^+$ $CD45RO^+$ T cells. *Immunobiology* 2013;**218**:90–95.

52 E. Svobodova, M. Krulova, A. Zajicova, *et al*. The role of mouse mesenchymal stromal cells in differentiation of naive T-cells into anti-inflammatory regulatory T-cell or proinflammatory helper T-cell 17 population. *Stem Cells Dev* 2012;**21**:901–910.

53 A. Nasef, A. Chapel, C. Mazurier, *et al*. Identification of IL-10 and TGF-β transcripts involved in the inhibition of T-lymphocyte proliferation during cell contact with human mesenchymal stromal cells. *Gene Expr* 2007;**13**:217–226.

54 M. M. Duffy, J. Pindjakova, S. A. Hanley, *et al*. Mesenchymal stem cell inhibition of T-helper 17 cell-differentiation is triggered by cell–cell contact and mediated by prostaglandin E2 via the EP4 receptor. *Eur J Immunol* 2011;**41**:2840–2851.

55 I. K. Jang, H. H. Yoon, M. S. Yang, *et al*. B7-H1 inhibits T cell proliferation through MHC class II in human mesenchymal stromal cells. *Transplant Proc* 2014;**46**:1638–1641.

56 A. Augello, R. Tasso, S. M. Negrini, *et al*. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;**35**:1482–1490.

57 A. Uccelli, L. Moretta, and V. Pistoia. Mesenchymal stromal cells in health and disease. *Nat Rev Immunol* 2008;**8**:726–736.

58 S. Beyth, Z. Borovsky, D. Mevorach, *et al*. Human mesenchymal stromal cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;**105**:2214–2219.

59 K. Le Blanc, I. Rasmusson, C. Götherström, *et al*. Mesenchymal stromal cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. *Scand J Immunol* 2004;**60**:307–315.

60 R. Meisel, A. Zibert, M. Laryea, *et al*. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**: 4619–4621.

61 B. J. Jones, G. Brooke, K. Atkinson, and S. J. McTaggart. Immunosuppression by placental indoleamine 2,3-dioxygenase: a role for mesenchymal stromal cells. *Placenta* 2007;**28**:1174–1181.

62 K. Nemeth, A. Leelahavanichkul, P. S. Yuen, *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

63 A. Nasef, N. Mathieu, A. Chapel, *et al*. Immunosuppressive effects of mesenchymal stromal cells: involvement of HLA-G. *Transplantation* 2007;**84**:231–237.

64 D. Chabannes, M. Hill, E. Merieau, *et al*. A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stromal cells. *Blood* 2007;**110**:3691–3694.

65 K. Sato, K. Ozaki, I. Oh, *et al*. Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stromal cells. *Blood* 2007;**109**:228–234.

66 J. H. Lim, J. S. Kim, I. H. Yoon, *et al*. Immunomodulation of delayed-type hypersensitivity responses by mesenchymal stromal cells is associated with bystander T cell apoptosis in the draining lymph node. *J Immunol* 2010;**185**:4022–4029.

67 G. Ren, L. Zhang, X. Zhao, *et al*. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**:141–150.

68 M. Sioud, A. Mobergslien, A. Boudabous, and Y. Floisand. Mesenchymal stem cell-mediated T cell suppression occurs through secreted galectins. *Int J Oncol* 2011;**38**:385–390.

69 F. Gieseke, A. Kruchen, N. Tzaribachev, *et al*. Proinflammatory stimuli induce galectin-9 in human mesenchymal stromal cells to suppress T-cell proliferation. *Eur J Immunol* 2013;**43**:2741–2749.

70 M. Sioud, A. Mobergslien, A. Boudabous, and Y. Floisand. Evidence for the involvement of galectin-3 in mesenchymal stem cell suppression of allogeneic T-cell proliferation. *Scand J Immunol* 2010;**71**:267–274.

71 Y. Lepelletier, S. Lecourt, A. Renand, *et al*. Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. *Stem Cells Dev* 2010;**19**:1075–1079.

72 K. Le Blanc, F. Frassoni, L. Ball, *et al*. Mesenchymal stromal cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**:1579–1586.

73 Y. Zhou, A. Day, S. Haykal, *et al*. Mesenchymal stromal cells augment $CD4^+$ and $CD8^+$ T-cell proliferation through a CCL2 pathway. *Cytotherapy* 2013;**15**:1195–1207.

74 M. Sajic, D. P. Hunt, W. Lee, *et al*. Mesenchymal stromal cells lack efficacy in the treatment of experimental autoimmune neuritis

despite *in vitro* inhibition of T-cell proliferation. *PLoS ONE* 2012;**7**: e30708.

75 E. Schurgers, H. Kelchtermans, T. Mitera, *et al*. Discrepancy between the *in vitro* and *in vivo* effects of murine mesenchymal stromal cells on T-cell proliferation and collagen-induced arthritis. *Arthritis Res Ther* 2010;**12**:R31.

76 M. Dominici, K. Le Blanc, I. Mueller, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

77 E. Ben-Ami, A. Miller, and S. Berrih-Aknin. T cells from autoimmune patients display reduced sensitivity to immunoregulation by mesenchymal stromal cells: role of IL-2. *Autoimmun Rev* 2014;**13**:187–196.

78 Z. Zhang, Y. Ding, W. Li, B. Song, and R. Yang. Interleukin-17A- or tumor necrosis factor α-mediated increase in proliferation of T cells cocultured with synovium-derived mesenchymal stromal cells in rheumatoid arthritis. *Arthritis Res Ther* 2013;**15**:R169.

79 M. D. Griffin, S. J. Elliman, E. Cahill, *et al*. Concise review: adult mesenchymal stromal cell therapy for inflammatory diseases: how well are we joining the dots? *Stem Cells* 2013;**31**:2033–2041.

80 A. Corcione, F. Benvenuto, E. Ferretti, *et al*. Human mesenchymal stromal cells modulate B-cell functions. *Blood* 2006;**107**:367–372.

81 I. Rasmusson, K. Le Blanc, B. Sundberg, and O. Ringdén. Mesenchymal stromal cells stimulate antibody secretion in human B cells. *Scand J Immunol* 2007;**65**:336–343.

82 S. Tabera, J. A. Pérez-Simón, M. Díez-Campelo, *et al*. The effect of mesenchymal stromal cells on the viability, proliferation and differentiation of B-lymphocytes. *Haematologica* 2008;**93**:1301–1309.

83 A. Ribeiro, P. Laranjeira, S. Mendes, *et al*. Mesenchymal stromal cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells. *Stem Cell Res Ther* 2013;**4**:125.

84 F. Schena, C. Gambini, A. Gregorio, *et al*. Interferon-γ-dependent inhibition of B cell activation by bone marrow-derived mesenchymal stromal cells in a murine model of systemic lupus erythematosus. *Arthritis Rheum* 2010;**62**:2776–2786.

85 Y. Peng, X. Chen, Q. Liu, *et al*. Alteration of naive and memory B-cell subset in chronic graft-versus-host disease patients after treatment with mesenchymal stromal cells. *Stem Cells Transl Med* 2014;**3**: 1023–1031.

86 M. Budoni, A. Fierabracci, R. Luciano, *et al*. The immunosuppressive effect of mesenchymal stromal cells on B lymphocytes is mediated by membrane vesicles. *Cell Transplant* 2013;**22**:369–379.

87 M. Ichii, K. Oritani, T. Yokota, *et al*. Regulation of human B lymphopoiesis by the transforming growth factor-β superfamily in a newly established coculture system using human mesenchymal stromal cells as a supportive microenvironment. *Exp Hematol* 2008;**36**:587–597.

88 S. Yoshioka, Y. Miura, H. Yao, *et al*. CCAAT/enhancer-binding protein β expressed by bone marrow mesenchymal stromal cells regulates early B-cell lymphopoiesis. *Stem Cells* 2014;**32**:730–740.

CHAPTER 35

# The role of mesenchymal stromal cells in the repair of acute organ injury*

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## 35.1 Effect of acute organ injury on the proliferative and functional activity of mesenchymal stromal cells

Acute organ injury markedly alters the microenvironment, which affects the proliferation, differentiation, and secretory activity of mesenchymal stromal cells (MSCs).

### 35.1.1 The effect of catecholamines on mesenchymal stromal cells

The pain accompanying acute organ injury activates the neuroendocrine system and, first and foremost, the hypophyseal portal system. This system is a system of blood vessels in the brain that connects the hypothalamus with the anterior pituitary. Its main function is the transport and exchange of hormones to allow fast communication between both glands. The *fenestrae* structure of the hypophyseal portal system needs only a small amount of hormones to allow a rapid exchange between the hypothalamus and the pituitary gland in order to stimulate an effect in the respective target organs in the body. Release of catecholamines directly affects

* The order of the authors' names indicates the relative contribution of each author to each section.

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MSCs by increasing the level of DNA synthesis through the activation of α-1 adrenergic receptors [1]. It has been demonstrated that physiological doses of epinephrine (adrenaline) and norepinephrine (noradrenaline) augment MSC proliferation *in vitro* [2]. However, concentrations of these stress hormones increase abruptly in acute organ injury, resulting in almost complete inhibition of MSC colony growth to the level of 4–5% of normal [2]. We suggest that this inhibition of MSC proliferation may be induced by an acute increase in interleukin (IL)-6 levels, the synthesis of which is increased as the catecholamine level rises.

Dasu *et al.* demonstrated that the pathways of activation of adrenoreceptors and toll-like receptors (TLRs) are closely interrelated [3]. Treatment of MSCs with epinephrine increases the β2 adrenergic receptor level and expression of β adrenergic receptor kinase protein, disturbs cell migration of bone-marrow-derived MSCs by 21% and enhances IL-6 synthesis tenfold. This effect was significantly reduced when MSCs were cultured in the presence of the β2 adrenergic receptor antagonist [3]. Furthermore, it has been demonstrated that MSCs can synthesize catecholamines and their level can be regulated by activation of TLR2. We hypothesize that elevation of epinephrine secretion by MSCs in acute organ injury and activation of the MSCs' TLR-signaling system will lead to high production of proinflammatory cytokines and inhibition of injury recovery.

As discussed in Section 35.1.2, changes in tissue oxygen ($O_2$) tension are another crucial factor affecting the proliferation, differentiation, and paracrine function of MSCs in acute organ injury. Thus, increased levels of stress hormones in acute organ damage have a direct effect on the proliferation of MSCs. The observed decrease in proliferative activity is attributed to a protective mechanism that prevents cell death in the acute phase of the injury.

### 35.1.2 The impact of hypoxia as a factor of acute injury on mesenchymal stromal cell proliferation

Acute organ injury is accompanied by a severe inflammatory response and microcirculatory and metabolic abnormalities, which almost always cause tissue hypoxia. The impact of this factor on the functional activity of MSCs is discussed herein.

According to the literature, $O_2$ tension ranges from as high as 12% in blood to as low as 1% in cartilage [4]. Oxygen tension in the bone marrow has been reported to vary from 3% to 7% [5]. It has been demonstrated that a 2-week cell culture at an $O_2$ concentration reduced to 2% stimulates strong proliferative activity of MSCs, resulting in an over 30-fold increase in the number of MSCs compared with those cultured under normoxic conditions [6]. The authors of this study showed that hypoxia also affected MSC morphology and the expression level of certain genes. Thus, enhanced expression of *OCT-4* and *HIF-2α* genes was clearly detected. The *OCT-4* level increased as early as 24 h under hypoxic conditions and reached its maximum level on day 3. Enhanced expression of *OCT-4* is indicative of the increased pluripotency level in embryonic stem cells, and this also appears to apply to adult multilineage inducible cells [7].

D'Ippolito *et al.* reported data suggesting that culture of marrow-isolated adult multilineage inducible cells derived from murine bone marrow at 3% $O_2$ level accelerated proliferation, which caused a threefold increase in cell number within 7 days of culturing [8]. These cellular changes were also accompanied by enhanced expression of *REX-1* (a gene expressed in blastocyst cells) and *OCT-4* (one of the genes expressed during embryogenesis). Berniakovich and Giorgio demonstrated that culturing MSCs derived from murine bone marrow for 7 days at 3% $O_2$ level enhanced *OCT-4* and *REX-1* expression levels four- and six-fold respectively [9].

Thus, under hypoxic conditions, MSCs may be able to retain an undifferentiated phenotype and allow self-renewal [10]. In contrast, another study demonstrated that hypoxia stimulated differentiation of rat MSCs along the osteogenic pathway [11]. Ren *et al.* demonstrated that expression of major histocompatibility complex (MHC) proteins is significantly enhanced by culturing murine MSCs in the presence of 8% $O_2$ [12], which in turn was indicative of intensive differentiation. It was also shown that culturing MSCs in the presence of 8% $O_2$ inhibited *OCT-4* expression in MSCs.

Such discrepancy in the data from these studies may be due to the heterogeneity of the MSC population used. Hence, Nagano *et al.* demonstrated that MSCs derived from human umbilical cord blood and capable of differentiating along the adipogenic and chondrogenic lineages were populations characterized by different activity of aldehyde dehydrogenase [13]. The cells with high activity of this enzyme exhibited a higher proliferation rate and better differentiation ability under hypoxic conditions. This subtype of cells had higher levels of synthesis of FLT-1, CXCR4, and angiopoetin-2 proteins. The initial difference in metabolic activity may offer an advantage for proliferation of a certain MSC subpopulation under hypoxic conditions.

Ren *et al.* also reported that, in murine bone-marrow-derived MSC cultures, two cell types could be distinguished morphologically: spindle-shaped cells and large flat cells. Furthermore, early colonies also contained very small round cells that rapidly self-renewed [12]. Preferential proliferation of large flat cells compared with other cell types was observed when $O_2$ tension in the culture was reduced to 8%.

A number of chemokines responsible for MSC migration to sites of inflammation are produced during the inflammatory response and concomitant hypoxia [14]. Migration or transplantation of MSCs into hypoxic tissues can be accompanied by activation of cellular apoptosis [15–17].

The level of growth factors maintaining cellular metabolism becomes the limiting factor under hypoxic conditions. Thus, MSC viability is reduced under hypoxic conditions if MSCs are cultured in a serum-free medium [17]. Cell culturing in a serum-free medium (serum deprivation) increases the degree of apoptosis. Its combination with hypoxia increases it even more. We hypothesize that growth factors that provide cell adaptation to the new environment are required for MSCs to survive under hypoxic conditions. MSCs have been shown to be able to

rearrange their metabolism when adjusting to new microenvironmental conditions.

Hypoxia-preconditioned MSCs showed increased expression of antiapoptotic factors, including BCL-2 and BCL-XL [18], thus reducing caspase-3 activity and blocking apoptosis. MSCs preconditioned in a hypoxic environment and transplanted into the heart reduced cardiomyocyte apoptosis by increasing BCL-2 production [19–21]. Lavrentieva *et al.* reported that reduction in $O_2$ tension during culture of human MSCs to 1.5% increased expression of hypoxia-inducible factor (HIF)-1-dependent genes such as *GLUT1*, *LDHA*, and *PDK1* [22]. A significantly higher glucose consumption by cells and increased lactate production was observed at 1.5% $O_2$. These metabolic changes reduced the MSC necrosis level under hypoxic conditions, while the apoptotic level did not change significantly.

HIF is one of the key transcription factors involved in adaptation of MSCs to reduced $O_2$ tension. This factor is inactivated at increased $O_2$ tension levels and is stabilized under hypoxic conditions [23]. HIF activation induces expression of the genes determining the pro-survival ability of MSCs under hypoxic conditions (in particular, glucose-6-phosphate transporter) [24].

Data concerning the impact of $O_2$ tension levels lower than 2% on MSC proliferation vary. Martin-Rendon *et al.* observed augmented proliferation even after short-term culture at 1.5 $pO_2$ [25]. Rosová *et al.* did not observe this effect [26], while Hung *et al.* reported reduced proliferation level during cell culturing at 1% $O_2$ [27].

Thus, hypoxia occurring during acute organ injury is a powerful factor that triggers the adaptation processes of MSCs. Changes in biochemical indicators and gene expression contribute to the conditions essential for the survival of MSCs in the acute phase of injury and create the necessary conditions for subsequent MSC proliferation in the regeneration required for healing of the acute injury.

### 35.1.3 Effect of hypoxia as a factor of acute injury on the paracrine function of mesenchymal stromal cells

Hypoxia also alters production of paracrine factors secreted by stromal cells. The activation of HIF-1α production induces transcription of angiogenic factors [28,29]. Ohnishi *et al.* demonstrated that expression of 135 genes (many of which regulate glycolysis and metabolism) was augmented more than threefold by culturing MSCs in 1% $O_2$ for 24 h compared with normoxic culture conditions. Such genes as *VEGF-D*, *PGF*, *PBEF1*, *HB-EGF*, and *MMP-9* are some of those involved in the proliferation of MSCs [30]. It has repeatedly been shown that hypoxia enhances production of such factors as vascular endothelial growth factor (VEGF), fibroblast-derived growth factor (FGF), hepatocyte growth factor (HGF), and insulin-like growth factor (IGF) by MSCs [31–35].

Hypoxia affects the production of trophic, pro-angiogenic and anti-inflammatory factors by MSCs, while the use of the conditioned medium (CM) obtained by culturing MSCs under hypoxic conditions is more effective for treating a number of pathologic conditions compared with medium obtained by culturing MSCs at normal $O_2$ tension [36–39]. We demonstrated that the CM obtained by culturing MSCs isolated from cluster of differentiation 1 (CD1) mice for 3 days, when combined with lysate of these cells, had a protective effect in mice with acetaminophen-induced acute liver failure. (CD1 is a family of glycoproteins expressed on the surface of various antigen-presenting cells. They are related to the class I MHC molecules and are involved in the presentation of lipid antigens to T cells. However, their precise function is unknown. CD1 mice lack group 1 CD1 molecules and instead have two copies of CD1d.) Acetominophen is paracetamol and causes acute liver failure in humans and animals if overdoses are taken. In this experiment, MSCs were cultured at normal $O_2$ tension (21%) and under hypoxic conditions (10%). Biochemical analysis demonstrated that MSC-CM cultured both under hypoxic and normoxic conditions in combination with lysate significantly reduced the degree of acute liver failure. Liver damage was assessed by measuring alanine transaminsase and aspartate aminotransferase levels in the blood; liver damage results in elevation of theses enzymes.

The CM and the lysate obtained from normoxic MSC cultures showed the highest level of efficacy in the early stage of liver damage, with the mean alanine transaminsase level being 2508 U/L in the control group, 54.025 U/L in the normoxic group, and 223.16 U/L in the hypoxic group; the mean aspartate aminotransferase levels were 1275.75 U/L in the control group, 105.95 U/L in the normoxic group, and 621.4 U/L in the hypoxic group. In contrast, at later stages of liver damage the enzyme levels decreased more significantly in animals that received a combination of lysate and CM obtained from MSCs cultured under hypoxic conditions.

Histological data are shown in Figures 35.1, 35.2, 35.3, and 35.4.

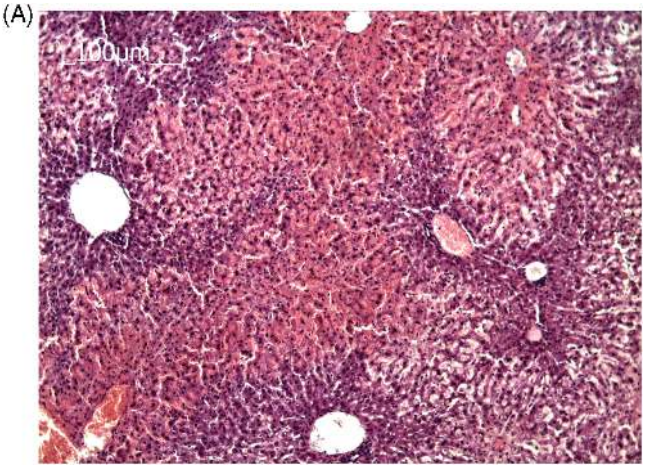
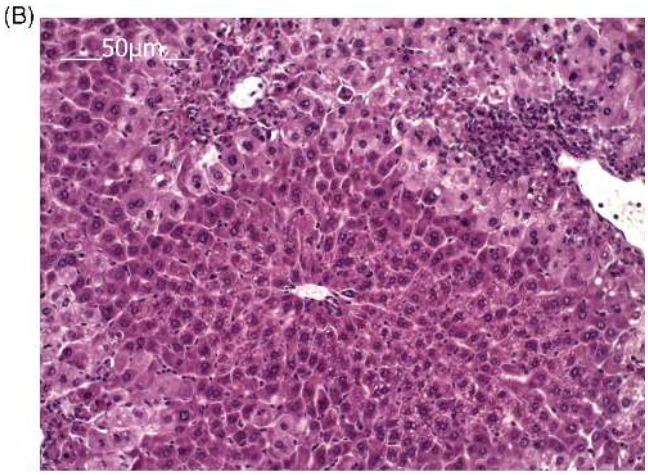



**Figure 35.1** Histopathologic findings in the liver of control mice after injection of acetaminophen (300 mg/kg) and Dulbecco's modified Eagle medium with 10% fetal bovine serum (1 mL). (A) 4 h: hepatocyte injury; necrosis of cells with plasma membrane disruption. (B) 24 h: adipose and protein dystrophy of hepatocytes; hepatocyte necrosis, leukocyte infiltration. (C) 48 h: adipose dystrophy of cells and chromatin margination; inflammatory infiltration.



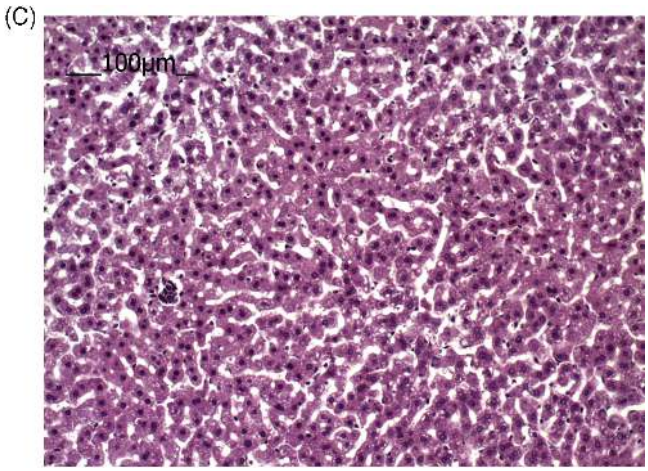
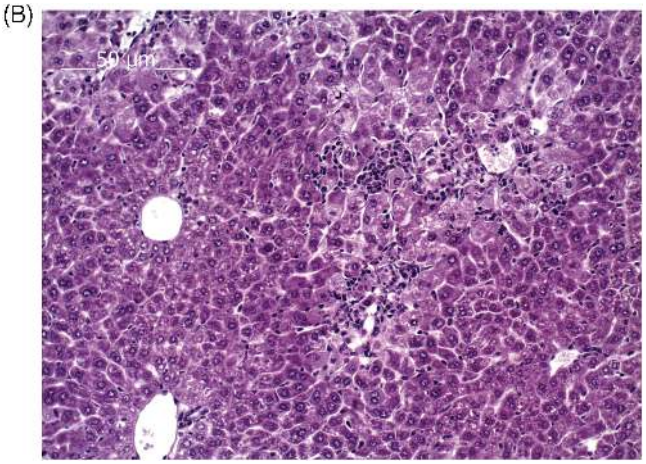




**Figure 35.1** (*Continued*)



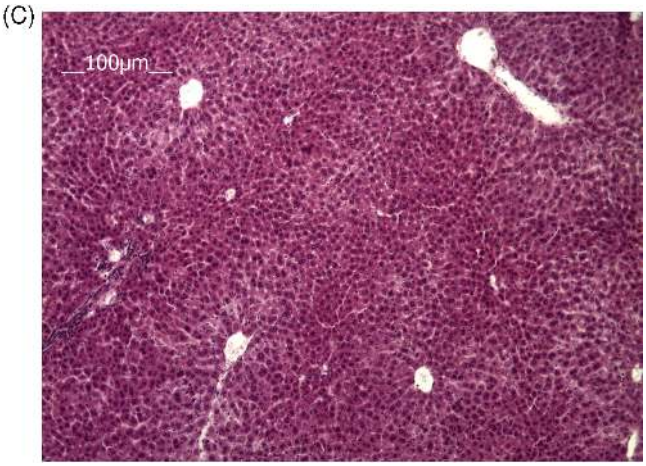




**Figure 35.2** (*Continued*)

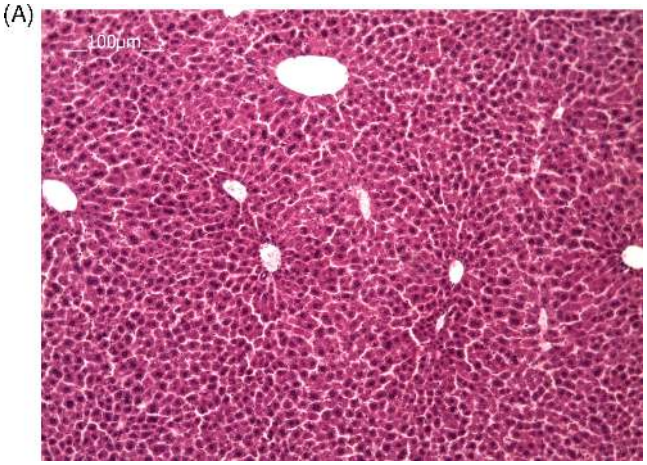



**Figure 35.2** Histopathologic findings in the liver of mice after injection of acetaminophen (300 mg/kg) and normoxic conditioned-medium MSCs (1 mL). (A) 4 h: hepatic lobules retain their trabecular structure; no signs of venous congestion or inflammatory infiltration are observed. (B) 24 h: the trabecular structure is retained in the periportal lobular regions; hepatocyte necrosis, leukocyte and macrophage infiltration. (C) 48 h: recovery of the injured tissues; hepatocyte dystrophy.

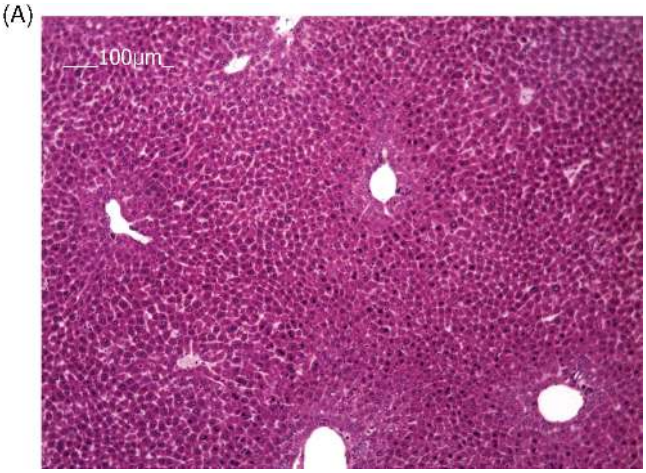
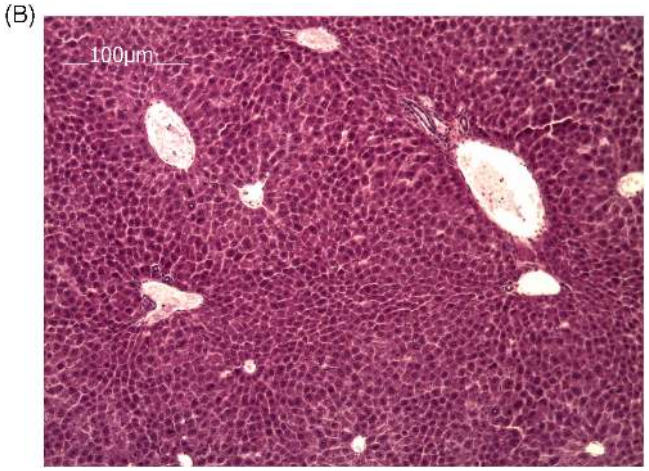



**Figure 35.3** Histopathologic findings in the liver of mice after injection of acetaminophen (300 mg/kg) and hypoxic conditioned-medium MSCs (1 mL). (A) 4 h: minimal tissue damage; no signs of circulation disruption or inflammatory infiltration were observed. (B) 24 h: macrovesicular adipose dystrophy in hepatocytes and cellular necrosis; no signs of inflammatory infiltration or circulation disruption were detected. (C) 48 h: no nuclear disruption is revealed; rather, regenerating cells with large hyperchromatic nuclei and binuclear hepatocytes were detected.



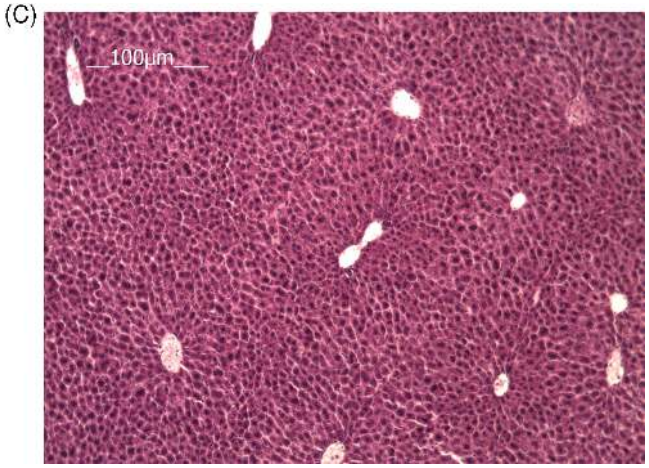




**Figure 35.3** (*Continued*)

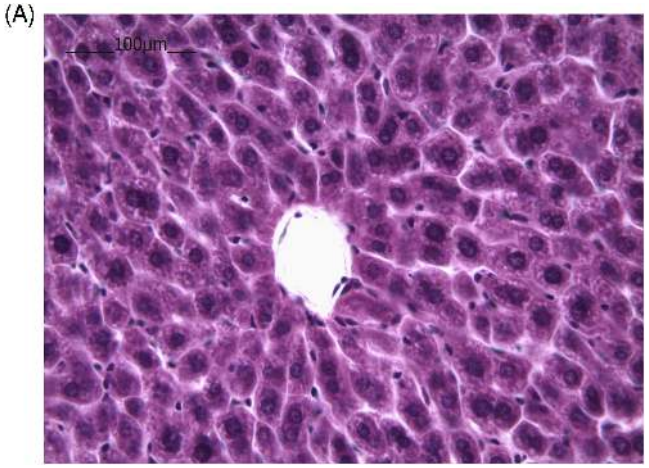



**Figure 35.4** Marked hepatocyte proliferative activity 48 h after administration of acetaminophen and hypoxic cMSC; (B) is a 20-fold magnified view of (A).

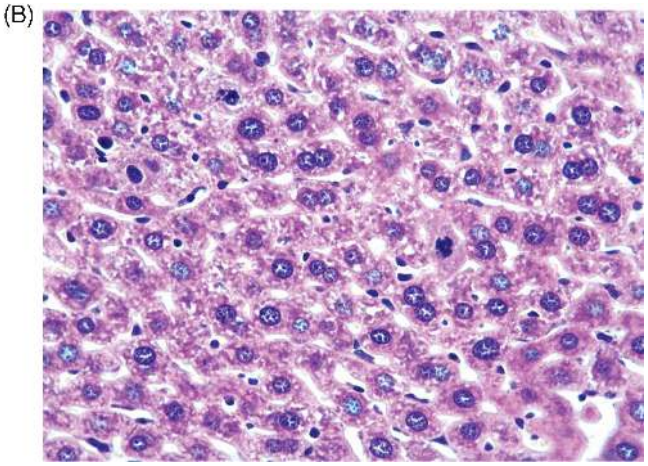



**Figure 35.4** (*Continued*)

Oxygen tension in tissues containing MSCs *in vivo* is known to vary from 1 to 7% depending on how far the tissues lie from blood vessels. Inflammation, edema, or disruption of blood supply can reduce $O_2$ tension to 0.4–2.3%. Hence, the conditions of MSC culture at $O_2$ tensions between 2.5 and 7% can be regarded as "physiological normoxia."

Centralization of blood flow takes place in the acute phase of injury due to neurohumoral transmission. Neurohumoral transmission is the transmission of a nervous impulse from neuron to neuron or from neuron to effector organ or tissue by means of a neurohumoral substance, such epinephrine, norepinephrine, acetylcholine, and possibly histamine, serotonin, and γ-aminobutyric acid. This results in reduction of blood flow in tissues containing stromal cells and the induction of hypoxia in these cells. A gradual decrease in $O_2$ tension to 2.5–5% might activate proliferation of stromal cells whose metabolic activity is best adapted to such conditions [13]. Analysis of published data allows the consideration of 2.5–3% $O_2$ level to be this point; the highest proliferative activity was observed by a number of groups under these conditions [6,8,40]. Hung *et al.* recorded either reduced MSC proliferation or no significant change after $O_2$ tension was reduced below 2.5% [27]. Studies using MSCs derived from rats, animals with the most active metabolism, demonstrated that an increase in $O_2$ tension to 5% enhanced proliferation by 40% [11]. A reduction in tissue $O_2$ tension to 0.4–1% is a direct death threat to cells, in which proliferative processes will be replaced by metabolic adaptation accompanied by enhanced expression of glucose transporter 1 and other molecules [22]. Maximum gene expression is observed at an $O_2$ tension of 1.5% compared with 2.5 and 5%.

Thus, changes in the production of paracrine factors by MSCs affected by hypoxia is aimed at minimizing tissue and cell damage and the creation of optimal conditions for subsequent regeneration.

### 35.1.4 Effect of tissue-specific proteins released after acute tissue injury on mesenchymal stromal cells

Acute injury induces a release of intracellular proteins liberated during cell and tissue degradation. It has been shown in a number of studies that these tissue-specific proteins can affect stromal cell differentiation along the appropriate lineage, ensuring recovery of the injured organ.

Choi *et al.* cultured bone-marrow-derived MSCs with pancreatic tissue extract and found that MSCs started expressing four hormones (insulin, glucagon, pancreatic polypeptide, and somatostatin) after 7 days. The cells also secreted insulin in response to supplementation of growth medium with glucose [41].

Chang *et al.* demonstrated that rat MSCs treated with proteins derived from post-infarction myocardium were capable of expressing cardiac-specific markers. The expression level was enhanced by using an extract from ischemic myocardium compared with that from uninfarcted myocardium [42]. The extract from ischemic tissue was found to contain higher transforming growth factor-β (TGF-β) and bone morphogenetic protein-2 (BMP-2) concentrations, while supplementation of growth medium with inhibitors of TGF-β or BMP-2 inhibitors blocked the expression of cardiac-specific proteins by MSCs.

Human MSCs treated with methylating agents, such as 5-azacitidine at a concentration of 10 μM, can differentiate along the cardiomyocyte lineage and express tissue-specific desmin, troponin I, sarco/endoplasmic reticulum $Ca^{2+}$-ATPase 2 (a calcium ATPase-type P-ATPase), and cardiac myosin heavy chain antigens. The same differentiation level could be obtained by culturing cells with an extract from neonatal rat cardiomyocytes [43].

Human cardiac explant-CM contains a high level of proteins capable of reprogramming the MSC genome and promoting their differentiation along the cardiomyocyte lineage. About 20 of these proteins are growth factors and cytokines [44]. *In vitro* supplementation of MSCs with this medium enhances their proliferative activity so that 3–5% of cells start to express cardiac-specific proteins. It should also be mentioned that an extract derived from injured brain tissue reduced *OKT-4* expression, enhanced *nestin* expression in MSCs, and increased the level of apoptosis in the cell culture. The extract derived from injured brain exhibited higher toxicity than that derived from healthy brain tissue [45].

Human macrophages are divided into M1 and M2 types. M1/M2 describes the two major and opposing activities of macrophages. M1 activity inhibits cell proliferation and causes tissue damage, while M2 activity promotes cell proliferation and tissue repair. The molecules primarily responsible for these "Fight" (nitric acid) or "Fix" (ornithine) activities both arise from arginine and via enzymatic pathways (inducible nitric oxide synthetase and arginase) that downregulate each other. The names M1 and M2 were chosen because M1 and M2 macrophages promote helper T cells type-1 and -2 responses respectively. M2 macrophages seem to play a crucial role in reprogramming the MSC genome induced by tissue-specific proteins released after acute tissue damage as they actively consume necrotic and apoptotic cells in the injured areas and secrete the anti-inflammatory cytokines TGF-β and IL-10 [46]. Furthermore, M2 macrophages release growth factors, such as IGF-1, in the injured area [47]. It has been demonstrated that IL-10 secreted by macrophages is required to sustain viability, differentiation, and fusion of mesoangioblast-like myogenic progenitors in toxin-induced skeletal muscle injury [48].

Additionally, injection of the CM from human placenta-derived MSCs increased the number of M2 macrophages near the wound [49]. Thus, tissue-specific factors released from damaged tissues is one of the signaling mechanisms underlying the tissue-specific regeneration process of MSCs.

## 35.2 Paracrine effect of mesenchymal stromal cells in acute organ injury

### 35.2.1 Background

The beneficial effect of MSCs in various pathological settings has been demonstrated in numerous experimental and clinical studies. Many years have passed since Friedenstein and his colleagues first isolated MSCs from bone marrow in 1968 and described them as a culture of fibroblast-like cells that could transdifferentiate into bone cells [50]. He used the term colony-forming unit–fibroblast (CFU-F) to describe colonies of these cells because of their superficial microscopic resemblance to fibroblasts.

The understanding of the mechanism of action of MSCs has significantly changed since then. While the effect of injecting MSCs was initially attributed to their ability to differentiate into cells of the surrounding tissue (and remains so today with regard to disorders of the musculoskeletal system), the paracrine mechanism (i.e., the ability of MSCs to synthesize and secrete a large number of proteins – including a number of cytokines and growth factors – that can affect the course of the pathological process in which the MSCs themselves are physically involved) is considered to be one of the key aspects of their mechanism of action. Paracrine signaling is a form of cell–cell communication in which a cell produces a signal to induce changes in *nearby* cells, altering the behavior or differentiation of those cells. It is different from endocrine signaling, in which one organ (e.g., the pituitary gland) secretes proteins, such as thyroid-stimulating hormone or adrenocorticotropic hormone, that have their effects on organs *distant* from the secretory organ – the thyroid gland in the case of thyroid-stimulating hormone and the adrenal glands in the case of adrenocorticotropic hormone.

It should be mentioned that the secretory activity of MSCs was reported in 1996 by Haynesworth *et al* [51]. and has been confirmed by a number of reports. First, injection of acellular MSC-CM has an effect similar to that of MSCs themselves. This has been demonstrated using preclinical models of injury of numerous organs, including kidney, heart, lung, liver, skeletal muscle, and urinary bladder [52–60]. Furthermore, the effect of

MSCs can often be observed as early as several hours after they are administered [61], which is insufficient time for them to differentiate. Thus, the short-term effect of MSC injection is most likely to be due to the paracrine mechanism. This aspect is especially relevant when considering a rapidly developing acute organ injury. Of note is the fact that the MSC migration pathways and the area of injury often do not coincide. For example, it has been demonstrated in the model of ischemia–reperfusion injury (IRI) in rats that most MSCs accumulated in the periportal zone, while the most profound antiapoptotic effect of injecting MSCs was observed in the centrolobular region, as shown by immunohistochemistry [62]. This clearly indicates that MSCs exhibit a paracrine effect. In this part of the chapter we will thoroughly describe the paracrine factors secreted by MSCs and discuss their main biological effect in acute organ injury.

### 35.2.2 Paracrine factors secreted by mesenchymal stromal cells

MSCs secrete a broad range of paracrine factors. Proteomic analysis has demonstrated that microvesicles (MVs) derived from MSCs contain over 730 different proteins [63]. These include cell adhesion and signaling molecules, cell surface receptors, ILs, and growth factors. We will discuss the production of MVs by MSCs. The key soluble paracrine factors that are likely responsible for the effects observed after MSC administration are shown in Table 35.1.

### 35.2.3 Immunosuppressive and anti-inflammatory effects of mesenchymal stromal cells

MSCs are both relatively nonimmunogenic as well as being actively suppressive of T cell, B cell, and natural killer cell activity. MSCs do not appear to be recognized by the immune system of immunocompetent recipients due, at least in part, to their lack of expression of MHC class II antigens as well as lack of the co-stimulatory molecules CD28 and CD80.

The immunosuppressive effect of MSCs has been known for some time. In 2002, Bartholomew *et al.* demonstrated that MSCs could reduce T-cell proliferation in response to an allogeneic stimulus by more than 50% [64]. The first description of the use of MSCs to treat acute graft-versus-host disease (GVHD) refractory to conventional therapy in humans was described in 2004 [65]. GVHD is mediated by donor T cells that recognize histocompatibility antigens present in the hematopoietic stem cell transplant recipient that are absent in the hematopoietic stem cell donor. In this report, haploidentical (maternal) MSC administration resulted in resolution of the GVHD in a recipient of an allogeneic hematopoietic stem cell transplant from an unrelated hematopoietic stem cell donor [65].

The anti-inflammatory effect of MSCs has also been demonstrated in preclinical models of endotoxic shock, lipopolysaccharide (LPS)-induced lung and liver injury, and in burns models [56,57,60,61,66,67].

It appears that both the immunosuppressive and anti-inflammatory effects of MSCs are mostly caused by the ability of MSCs to influence the expression and secretion of different cytokines. Hence, we will refer to these two effects together rather than discussing them separately. For example, the ability of MSCs to reduce concentrations of MCP-1 and MIP-1 [68,69], the powerful chemoattractants of the granulocyte-macrophage component of the innate immune system, abruptly inhibits the inflammatory infiltrate and thus reduces the severity of the inflammatory response, in particular during its early phase. MSCs secrete TGF-β and $PGE_2$, which inhibit synthesis of proinflammatory cytokines and T cell activation [70–73], thus downmodulating the inflammatory response in its later stages.

However, MSCs are also active producers of powerful proinflammatory agents, such as IL-1, IL-6, IL-8, IFN-γ, and TNF-α in

**Table 35.1** Most important paracrine factors secreted by MSCs.

| Group | Paracrine agents |
|---|---|
| Growth factors | TGF-β, HGF, VEGF, KGF, FGF, IGF-1, G-CSF, GM-CSF, IGF-2, M-CSF, PDGF-AA, EGF, NGF, BDNF, NT3, NT4 |
| Chemokines | MIP-2, MCP-1, MIP-1b, SDF-1, monokine induced by IFN-γ |
| Tumor necrosis factor | TNF-α,β |
| Interleukins | IL-1, IL-6, IL-8, IL-10 |
| Prostaglandins | $PGE_2$ |
| Cell adhesion molecule | ICAM-1 |
| Other proteins and enzymes | Adiponectin, IDO, MMP-9, IL-1 receptor antagonist, protein kinase B, sTNFR1, angiopoietin 1, TSG-6, NO, human leukocyte antigen-G5, IP-10, RANTES, IL-1RA |

BDNF: brain-derived neurotrophic factor; EGF: epidermal growth factor; G-CSF: granulocyte colony-stimulating factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; ICAM: intercellular adhesion molecule; IDO: indolamine 2,3-dioxygenase; IFN-γ: interferon-γ; IP-10: interferon-γ-induced protein 10; KGF: keratinocyte growth factor; IL-1RA: interleukin-1 receptor antagonist; M-CSF: macrophage colony-stimulating factor; MCP-1: monocyte chemoattractant protein 1; MIP: macrophage inflammatory protein; MMP: matrix metalloproteinase; NGF: nerve growth factor; NO: nitric oxide; NT: neurotrophin; PDGF: platelet-derived growth factor; $PGE_2$: prostaglandin $E_2$; RANTES: regulation on activation, normal T cell expressed and secreted; SDF: stromal cell-derived factor; sTNFR1: soluble tumor necrosis factor receptor 1; TNF: tumor necrosis factor; TSG-6: tumor necrosis factor α-induced protein-6.

some settings [74,75]. Furthermore, MSCs can also express TLRs, one of the main targets for proinflammatory cytokines, on their surface [75,76]. Thus, a discussion on MSCs and both pro- and anti-inflammatory cytokines is warranted.

TNF-α is a powerful inflammatory agent secreted by many cells (including MSCs), but mostly by monocytes and macrophages. This agent is pyrogenic, acts as a chemoattractant for most inflammatory cells, induces apoptosis and secretion of other proinflammatory agents (IL-1, IL-6, and IL-8), and inhibits lipoprotein lipase activity. In addition, TNF-α can independently cause lysis of certain cells [70]. TNF is often secreted by cells in response to activation of surface TLRs (TLR 1, 2, 4, and 6) by such ligands as LPS, fibrinogen, hyaluronic acid, and diacyl peptides. After surface TLRs become activated, nuclear factor kappa light chain-enhancer of activated B cells (NF-κB) is no longer controlled by its inhibiting agent the IκB kinase, which induces transcription of controlled genes and the production of proinflammatory cytokines.

The reported data on the ability of MSCs to affect TNF-α are variable. Lee *et al.* detected no statistically significant changes in the TNF-α level after administration of allogeneic human MSCs and MSC-CM using a model of *Escherichia coli* endotoxin-induced acute lung injury [66]. In contrast, the ability of MSCs to reduce TNF-α levels, either directly or indirectly, has been reported in most studies. Thus, a profound decrease in TNF-α produced by human umbilical-cord-blood-derived MSCs has been reported in a model of *in vitro* cisplatin-induced acute kidney injury [52]. The ability of MSCs to reduce the TNF-α level has also been demonstrated in preclinical models of colitis [77], antigen-induced arthritis [78], albumin-induced renal tubular inflammation [79], and acute lung injury [56,69].

Much attention has recently been focused on TSG-6. Increased expression of this gene enhances production of TNF-α-induced protein 6, which has anti-inflammatory properties. This protein activates inter-α-inhibitor, which, in turn, inhibits serine proteases, a key link in the development of inflammation [80].

It was demonstrated in a model of LPS-induced acute lung injury that administration of human MSCs by oropharyngeal aspiration 4 h after the exposure to LPS considerably reduced the inflammation and edema of pulmonary tissues. It was clearly demonstrated in the same study that secretion of TSG-6 by MSCs increased abruptly 12 h after their administration. Blockage of TSG-6 expression by RNA interference significantly reduced the anti-inflammatory activity of MSCs [69].

In a murine model of acute myocardial infarction the intravenous injection of human MSCs reduced the inflammatory response in the myocardium, in particular the activity of proinflammatory MMP-9, decreased the infarction border zone, and preserved myocardial function. Inhibition of TSG-6 significantly reduced this effect [81].

Wang *et al.* used a peritonitis model to demonstrate that the intraperitoneal injection of MSCs and MSC-CM significantly reduced the inflammatory cell infiltrate and cell adhesion. A significant increase in expression and secretion of TSG-6 was demonstrated. The outcome was substantially reduced by blockage of TSG-6 [82]. Similar data on the effect of TSG-6 were also obtained in a rat model of chemical burns of the cornea [83].

Albumin-induced inflammation of tubular epithelium was modeled *in vitro* in another study. Bone-marrow-derived MSCs were cultured together with the cells of the renal proximal tubules. The investigators demonstrated high expression of TSG-6, which was activated by adding human serum albumin to the medium. However, in this case the enhanced expression of TSG-6 reduced the level of such factors as α-smooth muscle actin, fibronectin, and type IV collagen. Blockade of TSG-6 increased the level of these factors. Thus, TSG-6 was regarded in this study as an antifibrotic agent rather than an anti-inflammatory one [79].

sTNFR1 is believed to play a crucial role in TNF neutralization. The circulating receptor is capable of binding TNF-α, thus preventing its proinflammatory effect [84]. Furthermore, data indicate that sTNFR1 facilitates TGF-β1 formation, which reduces the proliferation of T cells [56]. Hence, the similarity between the anti-inflammatory and immunosuppressive effect of MSCs was again demonstrated. Yagi *et al.* clearly demonstrated that bone-marrow-derived MSCs can secrete sTNFR1. Moreover, this secretory activity considerably increased LPS-induced inflammation. Administration of anti-sTNFR1 monoclonal antibodies reduced the positive *in vivo* effect of MSCs, while the level of proinflammatory cytokines (TNF-α, IL-6, IFN-γ) in animal serum increased [56]. The authors thought that a negative feedback mechanism was induced in this case: increased concentration of TNF-α or LPS led to increased production of sTNFR1.

Another study compared the ability to secrete sTNFR1 between bone-marrow-derived MSCs and adipose-tissue-derived MSCs [67]. Bone-marrow-derived MSCs were shown to secrete a higher level of sTNFR1 than adipose-tissue-derived MSCs. Furthermore, it was demonstrated that neutralization of sTNFR1 did not significantly reduce the positive effect of bone-marrow-derived MSCs. Thus, the role of sTNFR1 in the anti-inflammatory effect remains unclear.

The role of different types of TLRs in the anti-inflammatory properties of MSCs is controversial. MSCs express several different types of TLRs (e.g., TLR 2, 3, and 4) [85]. Enhanced expression of certain TLRs in MSCs can be stimulated by adding the corresponding agents (e.g., LPS to stimulate expression of TLR 4 or polyinosinic:polycytidylic acid (polyI:C) to stimulate expression of TLR 3). Stimulation of monocytes with TLR agonists caused their transformation into the M1 phenotype involved in the inflammatory response, while stimulation of IL-4 gave rise to monocytes with the M2 phenotype, which exhibit anti-inflammatory properties [86]. Waterman *et al.* hypothesized that this polarization is possible for MSCs as well. However, the MSC response depended on the type of TLR (TLR 3 or TLR 4) the MSCs expressed. Thus, stimulation of TLR 4 expression induced greater secretion of proinflammatory cytokines (IL-6, IL-8), while TLR 3 expression enhanced secretion of anti-

inflammatory factors (IP-10, RANTES, IL-1RA) and, to a lesser degree, IL-4 and IL-10 [76]. It was also demonstrated in this study that MSCs with enhanced expression of TLR 3 significantly reduced secretion of TGF-β1 and TGF-β3, two key immunosuppressive factors, which may be involved in a negative feedback mechanism.

Hwang *et al.* studied the expression of different types of TLRs in MSCs derived from human nasal mucosa. They demonstrated that these cells mostly expressed TLR 3 and TLR 4, while expressing TLR 2 and TLR 5 to a lesser extent. In turn, exposure to TLR agonists (LPS and polyI:C) did not significantly increase expression of TLR 3 and TLR 5 in these cells but enhanced expression of TLR 2 and TLR 4. Exposure to LPS stimulated these MSCs to secrete both pro- and anti-inflammatory cytokines, while exposure to polyI:C had no effect on secretion of these cytokines by the cells, demonstrating that TLR 4 was more active than TLR 3 in this case [75].

The respiratory tract mucous membranes are exposed to significant antigenic stresses *in vivo*, which may induce phenotypic changes in cells in the mucosa, including MSCs. High expression of TLR 4 in the MSCs of nasal mucosa is likely associated with the need to provide a timely immune response to antigenic irritants.

The ability of MSCs to modulate the level of anti-inflammatory cytokines is also important. IL-10 is a powerful anti-inflammatory agent whose effect is mediated by a suppressive action on the effector functions of macrophages, T cells, dendritic cells, and natural killer cells. It also inhibits synthesis of some proinflammatory cytokines. Yagi *et al.* detected a threefold increase in the level of IL-10 gene expression in MSCs co-cultured with "inflammatory" serum derived from animals with an LPS-induced inflammatory syndrome or with thermal burns compared with control animals [60]. Danchuk *et al.* also found an increased level of IL-10 using the LPS-induced lung injury model [69]. Qui *et al.* demonstrated that administration of human umbilical cord-derived MSCs increased IL-10 and TGF-β levels in a rat model of cardiac transplantation [87]. An increased IL-10 level due to the effect of MSCs was also found in the model of ischemia–reperfusion-induced kidney injury, which will be discussed later in this chapter [88].

Peng *et al.* studied the effect of MSCs in patients with chronic GVHD and found that $CD5^+$ B cells in these patients showed greater gene expression for IL-10 after MSCs had been administered. IL-10 secretion is likely one of the key factors responsible for the clinical effect of MSCs [89].

It was shown in another study that co-culturing MSCs with peripheral blood mononuclear cells increased IL-10 mRNA production by mononuclear cells. An increased level of another anti-inflammatory cytokine, IL-4, was also observed [90]. Nevertheless, a number of authors reported no significant changes in IL-10 gene values when using MSCs or MSC-CM in different models *ex vivo* and *in vitro* [66,91,92]. Production of this factor may depends on external factors and medium conditions.

In the studies just described [89–92] and in many other studies, an important role has been attributed to induction of IDO secretion by MSCs. Furthermore, stimulation of MSCs with proinflammatory agents such as IFN-γ significantly enhanced secretion of this factor [93]. This enzyme induces L-tryptophan degradation via a kynurenine pathway, thus depriving T cells of an essential amino acid, thereby starving them to death and exerting a strong immunosuppressive and anti-inflammatory effect.

In another study, MSCs were cultured in medium supplemented with 20% synovial fluid collected from three groups of volunteers: those without any pathologies of joint disease, those with osteoarthritis, and those with rheumatoid arthritis. Under these conditions, expression levels of IDO mRNA and IL-6 were increased in MSCs in all three groups. Interestingly, however, expression of these factors after synovial fluid from patients with osteoarthritis and rheumatoid arthritis had been added to the culture was much higher. Furthermore, the MSC-CM derived from MSCs cultured in this manner profoundly reduced proliferation of $CD3^+$ $CD28^+$ T lymphocytes [94]. However, it was also demonstrated that MSCs can inhibit IDO synthesis by other cells. Thus, expression of this agent was enhanced in corneal epithelial cells stimulated by IFN-γ and TNF-α, while co-culturing these cells with bone-marrow-derived MSCs noticeably suppressed expression [95].

The secretion of another key immunosuppressive factor, TGF-β, by MSCs has also been described [95]. In particular, stimulation of bone-marrow-derived MSCs by IFN-γ or TNF-α abruptly increased secretion of this cytokine. The high secretory activity of MSCs with respect to TGF-β is associated with reduced expression of IDO by cells of the surrounding tissue. TGF-β has long been known for its powerful immunosuppressive activity and, in particular, its ability to inhibit proliferation and activation of lymphocytes and macrophages. MSCs can produce different amounts of this agent [87,96]. Expression of this factor is enhanced with inflammation and can mediate, at least in part, the anti-inflammatory effect of MSCs.

MSCs secrete a number of other anti-inflammatory agents, including $PGE_2$ [72], adiponectin (which can reduce TNF-α expression in adipose-tissue-derived MSCs), HGF, IGF, and endothelial nitrous oxide synthase [88].

The anti-inflammatory effect of HGF has been demonstrated in the preclinical model of albumin-induced renal tubular inflammation, in which it was found to inhibit transcription of TNF-α and the chemokines CCL-2 and CCL-5. The anti-inflammatory effect of MSCs notably decreased when HGF was neutralized [79].

The IGF family also have anti-inflammatory properties. In the LPS-induced lung injury model, IGF-2 contributed to the emergence of the anti-inflammatory M2 macrophage population, thus reducing the severity of inflammation [47].

Despite the fact that MSCs can produce some proinflammatory cytokines, it has been demonstrated in most studies that administration of MSCs reduces the total level of proinflammatory cytokines. It has been shown using various models that administration

of MSCs or MSC-CM profoundly reduced the expression and concentration of the following inflammatory agents: IL-1α and β, IL-6, IL-17, IFN-γ, G-CSF, GM-CSF, MIP-2α, and MCP-1 [52,56,60,68]. Secretion by MSCs of antagonists of some inflammatory cytokine receptors is likely one of the ways by which MSCs mediate their immune suppressive and anti-inflammatory properties. Thus, secretion of IL-1 receptor antagonist (IL-1RA) by MSCs increased more than fivefold after LPS was administered, although the basal expression level of this cytokine is low [69]. Secretion of IL-1RA by MSCs is significantly enhanced by culturing them in the presence of TNF, IFN-γ, and IL-1β [97].

IL-6 at high concentrations also appears to have an anti-inflammatory effect. Interestingly, intratracheal administration of 200 ng of IL-6 reduced the severity of inflammation in acute lung injury, decreased IL-1β, C–X–C motif chemokine ligand (CXCL)1, and TNF-α concentrations, and inhibited activity of lung myeloperoxidase, while it behaved as a typical proinflammatory cytokine when injected at the lower dose of 25 ng [98]. Since MSCs can produce high concentrations of IL-6 in inflammation, this factor can be regarded as an anti-inflammatory agent that reduces the levels of other proinflammatory cytokines.

MSCs can also reduce concentrations of granulocyte-macrophage chemoattractants, including MCP-1 and neutrophil-activation factor-3 (KC) [69].

The paracrine activity of MSCs may depend on different factors, including their tissue of origin and the health, gender, and age of the MSC donor. Crisostomo *et al.* studied the secretion of various proteins by MSCs *in vivo*. The level of VEGF secretion in female rats after activation of MSCs with LPS or hypoxia was higher in female rats, while the levels of TNF-α and IL-6 secretion were significantly lower than those in male rats [99]. Thus, MSCs from female rats exhibited lower proinflammatory activity and increased proliferative and regenerative activity compared with male rats. The authors hypothesized that estrogens might contribute to this effect. Exogenous estrogen has been reported to increase the activity of MSCs [100]. MSCs have the estrogen receptor alpha, which probably plays a role in their growth and differentiation [101], while mitogen-activated protein kinases (MAPKs) and cyclin-dependent kinases can mediate the proliferative effect of estrogens on murine embryonic stem cells [102].

We have shed light only on some tentative mechanisms of the immune suppressive and anti-inflammatory effect of MSCs. Nevertheless, we can infer that these abilities are mediated both by the secretion of anti-inflammatory and immunosuppressive agents by MSCs and by the influence of MSCs on the surrounding cells as they inhibit synthesis or reception of proinflammatory agents. In both settings, paracrine mechanisms are involved [71,72,103–107].

### 35.2.4 The pro-angiogenic and tissue regenerative effects of mesenchymal stromal cells in acute organ injury

In this section we will discuss the pro-angiogenic and tissue regenerative effects of MSCs as a component of their paracrine activities and will not focus on *de novo* differentiation of MSCs into cells of the appropriate mesodermal lineage, since it is paracrine mechanisms that are responsible for the early stages of angiogenesis and regeneration after MSCs are administered. Administration of MSCs caused enhanced proliferative activity [52] and angiogenesis [107] several days after acute organ injury had been induced.

It has been demonstrated in many studies that MSCs secrete significant levels of growth factors, including VEGF, HGF, FGF, heparin-binding EGF-like growth factor (HB-EGF), and placental growth factor (PLGF), which can stimulate angiogenesis in addition to induction of proliferation [108]. Furthermore, as with the secretion by MSCs of anti-inflammatory cytokines, secretion of these growth factors is in a dynamic equilibrium and can change in response to differing ambient conditions. An increase in secretion of cytokines by umbilical-cord-blood-derived MSCs was observed in response to cisplatin-induced injury: VEGF (fivefold), HGF (12-fold), FGF (twofold), and HB-EGF (sevenfold) [52]. It was noted in another study of bone-marrow-derived MSCs that expression of HGF, NGF, and SDF-1 significantly increased in response to administration of LPS, while expression of VEGF, NT3, and NT4 decreased [74]. Thus, the degree of growth factor secretion can vary depending on the injuring agent, health status of the MSC donor [74], and origin of the MSCs.

VEGF and HGF appear to play a crucial role in the regenerative and protective effects of MSCs, as well as in angiogenesis. High levels of these factors are observed with MSC-CM. When the effect of these factors was blocked by an appropriate monoclonal antibody, the effect of MSCs and MSC-CM was significantly reduced and cell survival *in vitro* decreased [68]. It has been demonstrated for angiogenesis that culturing umbilical-vein-derived endothelial cells in the absence of HGF significantly reduces their proliferative activity. The same effect is observed when HGF-depleted MSC-CM is added to an endothelial cell culture, while CM from intact MSCs stimulated endothelial proliferation [109].

The role of VEGF in angiogenesis cannot be overemphasized. Wu *et al.* demonstrated that the use of MSCs significantly accelerated wound recovery due to enhanced angiogenesis. A significant increase in VEGF expression by MSCs was demonstrated and was considered the most likely reason for the enhanced angiogenesis [107].

The preclinical model of cyclophosphamide-induced acute ovarian injury was used in another study. Cyclophosphamide abruptly reduced the $CD34^+$ cell count, suggesting inhibition of angiogenesis, while administration of adipose-tissue-derived MSCs considerably increased the survival rate of the cells compared with the untreated control group and with administration of fibroblasts. Cells expressing CD34 are normally found on early hematopoietic progenitor cells in the umbilical cord and bone marrow, a subset of mesenchymal stem cells, endothelial progenitor cells, and endothelial cells of blood vessels. It was also demonstrated in this study that MSCs secreted high levels of VEGF, IGF-1, and HGF, both *in vitro* and when transplanted

into the ovary [110]. Administration of these growth factors without MSCs was less effective.

It was shown in another study that MSC-CM contained high levels of VEGF, basic fibroblast growth factor (bFGF), PLGF, and MCP-1 and markedly enhanced proliferation of endothelium and smooth muscle cells. VEGF blockade inhibited endothelial proliferation, while FGF blockade inhibited proliferation of smooth muscle cells [108].

Genetic modification enables the regulation of production of some growth factors by MSCs. Thus, MSCs overexpressing GATA-4 produced more VEGF and IGF-1 than unmodified MSCs and contributed to a greater extent to the proliferation of endothelial cells, increased capillary density, and increased blood flow rate in a model of myocardial infarction. Neutralization of VEGF and IGF-1 significantly reduced the angiogenic effect of MSCs [111]. The *GATA-4* gene encodes a member of the GATA family of zinc finger transcription factors. Members of this family recognize the *GATA* motif that is present in the promoters of many genes. GATA-4 protein is thought to regulate genes involved in embryogenesis and in myocardial differentiation and function.

MSC-CM was also found to contain high levels of angipoietin (Ang)-1. This protein plays a crucial role in blood vessel formation and maturation and regulates the relationship between the endothelium and the extracellular matrix. On the contrary, Ang-2 is an Ang-1 antagonist as it inhibits binding of Ang-1 to the tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE-2) receptor. An increased Ang-1 level compared with the untreated control was detected in the lysate derived from wounds treated with MSC-CM, while the Ang-2 level remained intact. Hence, the Ang-1/Ang-2 ratio increased, which indirectly indicated induction of angiogenesis [107].

*In vitro* experiments have shown that co-culturing MSCs with endothelium noticeably enhances endothelial proliferation and facilitates formation of new capillaries. Endothelium is known to synthesize high levels of PDGF, while MSCs express receptors to this factor. This may be one of the mechanisms of interaction between the endothelium and MSCs [110].

However, it has also been demonstrated in the chemical corneal burn injury model that MSCs and MSC-CM can exhibit a negative effect on angiogenesis. The use of MSCs resulted in regression of vascularization [111], high expression of thrombospondin 1, an agent that induces endothelial apoptosis and reduces VEGF production. Reduced expression of the pro-angiogenic factor MMP-2 was also detected.

Thus, there is some disparity of results in studies of the angiogenic effect of MSCs, which seem related to the preclinical model employed.

A study be Lee *et al.* reported a profound decrease in the protective effect of MSCs after keratinocyte growth factor (KGF) knockdown by RNA interference. This was manifested as increased alveolar fluid clearance in the lungs. The effect was restored when recombinant KGF was added. KGF can increase the expression of epithelial sodium channels on the apical epithelial membrane, thus reducing the alveolar fluid level. Amiloride, a specific blocker of these channels, also reduced the effect of MSCs and MSC-CM, thus confirming the hypothesis concerning the mechanism of action of KGF [66].

The activity of growth factors derived from MSCs plays a crucial role in their protective effect. It is not only restricted to the regenerative and angiogenic effects. As mentioned earlier, HGF and IGF-1 exhibit anti-inflammatory properties. Some investigators believe that growth factors exhibit an antiapoptotic effect. This is discussed next.

### 35.2.5 The antiapoptotic activity of mesenchymal stromal cells

Kumar *et al.* induced apoptosis of human dermal microvascular endothelial cells by γ-radiation and found that treatment with VEGF significantly reduced the degree of apoptosis. The VEGF-treated endothelial cells showed markedly increased expression of B-cell lymphoma (Bcl)-2, an antiapoptotic agent, while expression of Bcl-2 in untreated cells subjected to radiation significantly decreased. Addition of phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) inhibitors to the VEGF-treated cell culture reduced Bcl-2 expression. This fact indicates that VEGF inhibits apoptosis by affecting the PI3K–Akt–Bcl-2 signaling pathway [112]. It was also demonstrated using the model of cisplatin-induced acute kidney failure that treatment with anti-VEGF antibodies reduced expression of protein kinase B (Akt), a powerful antiapoptotic agent [68].

The antiapoptotic effect of Akt is related to its ability to phosphorylate the Bcl-associated death promoter (BAD). BAD can form pores in the outer mitochondrial membrane, which results in cytochrome c release from mitochondria into the cytoplasm and activation of the cytoplasmic caspase cascade, causing cell death. When phosphorylated, BAD is dissociated and loses its pro-apoptotic activity [113].

The effect of MSCs on Akt is unlikely to be the only pathway by which they mediate inhibition of apoptosis. Oxidative stress is known to be another powerful factor inducing apoptosis [114]. MSCs and some MSC-secreted growth factors, in particular HGF, can inhibit oxidative stress by normalizing the functional activity of mitochondria and oxidative enzymes [115,116].

Despite the fact that its mechanism of action still remains subject to debate, the antiapoptotic effect of MSCs has been repeatedly demonstrated in a number of studies. Thus, Yin *et al.* used a rat preclinical model of acute spinal cord IRI to demonstrate that transplantation of bone-marrow-derived MSCs restored motor function to the forelegs [117]. The effect was observed as early as 4 days after the transplant. Function was almost completely restored by day 7 using the Basso, Beattie, and Bresnahan locomotor rating scale for evaluation. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining revealed a significantly smaller number of apoptotic cells in animals that received MSCs. (TUNEL is a method for detecting DNA fragmentation by labeling the terminal end of nucleic acids and is a common method for detecting DNA fragmentation

that results from apoptotic signaling cascades.) Reduction of apoptosis was also confirmed by immunohistochemistry (decreased caspase-3 level and the BAX/Bcl-2 ratio – BAX is Bcl-2-associated X protein).

The effect of MSCs on apoptosis has been studied in kidney, liver, and lung tissues [53,54,60,68]. Eliopoulos *et al.* used the model of cisplatin-induced acute kidney injury to demonstrate that MSCs both reduced the caspase-3 level and enhanced expression of the proliferation marker Ki-67 [68]. A significant reduction in caspase-3 level and degree of apoptosis was also revealed in a model of adriamycin-induced chronic renal failure [118]. While the antiapoptotic effect of MSCs in the model of acute renal injury could be detected as soon as several days after the injury, a model of chronic renal failure has shown that the effect of MSC administration persisted for 12 weeks.

Yagi *et al.* demonstrated that administration of MSCs noticeably reduced the number of apoptotic cells, and Akt overexpression was observed, after MSCs were introduced into the inflammatory medium (when "inflammatory serum" was added) in models of LPS-induced acute respiratory failure and thermal burns [60]. The antiapoptotic effect of human umbilical-cord-blood-derived MSCs in a model of acute optic nerve injury was observed at an early stage: caspase-3 level was assessed and TUNEL staining was performed. However, this effect was transient, since no differences were observed between the experimental and control groups at a later stage after the injury [119].

Thus, it is clear that MSCs exhibit an anti-apoptotic effect. This effect is most likely based on several pathways including the ability of MSCs to affect the PI3K–Akt–Bcl-2 signaling pathway and their ability to suppress oxidative stress.

### 35.2.6 Mesenchymal stromal-cell-derived microvesicles: an essential part of the paracrine mechanism

Secretion of growth factors and cytokines is a key property of MSCs and contributes to the positive effect of MSCs in a number of disorders, except for those of the musculoskeletal system where they are able to mediate their effect by transdifferentiation to the tissue into which they are injected.

The concept of the paracrine effect of MSCs now includes their ability to secrete MVs into the surrounding environment. MVs are exosomes 50–100 nm in size that contain a broad range of protein molecules and lipids as well as nucleic acids, with mRNAs and microRNAs being of particular interest. The most probable precursors of MVs are early endosomes and/or Golgi vesicles. Internal membrane folds are subsequently formed in them, resulting in formation of a large number of intraluminal vesicles inside the endosome. This complex is known as a multivesicular body. The membrane of the multivesicular body then fuses with the outer membrane of the MSC. Numerous intraluminal vesicles enter the intercellular medium, where they are referred to as MVs [120].

The role of MVs made by MSCs has not been completely clarified. However, a number of studies have been published demonstrating that introduction of isolated MVs can reduce the level of organ injury and see Chapters 1, 20, 38 and 57.

Thus, Bruno and colleagues demonstrated that MVs isolated from bone-marrow-derived MSCs significantly reduced mortality after cisplatin-induced kidney injury, decreased a histological abnormalities, and resulted in improved renal function as measured by reduced blood creatinine and urea levels compared with the untreated control animals [121]. Repeated administration of MVs had a greater effect than a single administration. However, the positive effect of introducing MVs was reduced after they had been subjected to long-term treatment with RNase, which suggests that mRNA and probably microRNAs embedded in MVs play a critical role in the mediation of the effect of the MVs. RNase concentration was significantly higher than physiological concentration, and the microvesicular membrane failed to protect RNA against cleavage [122,123]. A positive effect of MVs has also been demonstrated in other models of acute kidney injury, including those caused by glycerol administration [122], gentamicin administration [124], and IRI [123].

The role of MVs has also been demonstrated in injury models involving other organs. Herrera *et al.* found that MVs isolated from human hepatic stromal cells induced hepatocyte proliferation, reduced apoptosis *in vitro*, and contributed to regeneration and recovery of liver function after partial (70%) hepatectomy [125]. No effect was observed if MVs were treated with RNase. A protective effect from the administration of MSC MVs was demonstrated in other preclinical models of liver injury, including those caused by administration of CCL4, acetaminophen, and hydrogen peroxide [125,126]. It was shown that MVs enhance proliferation, manifested as enhanced expression levels of proliferating cell nuclear antigen (PCNA), which is a DNA clamp that acts as a processivity factor for DNA polymerase δ in eukaryotic cells and is essential for replication, as well as on cyclin D1. Additionally, apoptosis was decreased through increasing expression of the antiapoptotic factor Bcl-xL [126].

Lai *et al.* demonstrated that MVs reduced infarct size in a model of myocardial IRI in mice [127], and Bian *et al.* reported that MVs stimulated angiogenesis after myocardial infarction [128]. There are also studies confirming the protective effect of MVs in a model of lung injury [129].

Thus, it is clear that MVs play an important role in the activity of MSCs. However, the composition of MSC MVs has been analyzed in only a few studies. Kim *et al.* conducted a proteomic analysis of isolated MVs and found that they contained over 730 different proteins [63]. Most of these proteins are typically found in all types of MVs. However, 122 proteins detected in MVs were characteristic of MSCs. For example, MVs from MSCs had positive markers typical of those of MSCs, including CD13, CD29, CD44, CD73, and CD105, as well as some markers that have variable expression in MSCs, such as CD10. Fifty-five proteins (8%) revealed in MSC MVs were also found in MSC-CM.

Cell surface receptors (such as platelet-derived growth factor-β, epidermal growth factor receptor, and urokinase-type plasminogen activator receptor), which have a significant effect on

cell differentiation and proliferation and enhance mobilization and migration of MSCs, may play a key role in the therapeutic effect of MSCs. Signaling molecules such as RAS (a family of GTPases), MAPK1 (a protein kinase), RHO (also a family of GTPases, of which Cdc42 is a member) also play a significant role. These signaling pathways eventually enhance MSC proliferation and differentiation. The presence of molecules involved in cell adhesion, including fibronectin 1, ezrin, IQGAP1, CD47 (also known as integrin associated protein), galectins 1 and 3, and integrins, also indicates that MVs contribute to cell adhesion, migration, endothelial proliferation, angiogenesis, and vascular remodeling.

However, the main role of MVs is now attributed to the mRNAs and microRNAs contained within them. Ratajczak *et al.* demonstrated that embryonic stem cells can transfer mRNAs into other cells [130], while Chen *et al.* showed that MVs derived from MSCs were enriched in pre-microRNAs. mRNA and microRNA molecules penetrate target cells, thus affecting the cell's genome and changing the expression of various genes within the cell [131]. In this way, they cause critical changes in cell function. Migration of microRNA into a cell causes the phenomenon of RNA interference, the formation of complementary complexes between the mRNAs of the cell and microRNAs. Binding of the mRNA to microRNA results in inhibition of translation of the genes encoded in the region involved in the binding. The mRNA–microRNA complex is rapidly degraded by the cell [132]. It has also been reported that microRNAs interact directly with the DNA of the target cell [133]. MVs are likely excellent carriers of microRNAs and mRNAs, since they protect them against RNase activity. Some data are available indicating that MVs can be found in most fluids of the body [120].

There is presumably a mechanism for the selection of microRNAs to be transferred to MVs. Collino *et al.* found that, while MSCs and the MVs derived from them contain appreciably high levels of microRNAs, some microRNAs are detected only in MVs but not in MSCs [134]. The selection mechanism remains unclear. However, it is likely that microRNAs not transferred to MVs are destroyed by intracellular RNase.

Bruno *et al.* used the preclinical model of cisplatin-induced kidney injury to confirm that MV transfer mRNAs to the tubular cells and that proteins are translated from the transferred mRNAs. In this study, MVs were isolated from human bone-marrow-derived MSCs and carried the polymerase (RNA) II (DNA-directed) polypeptide E (*POLR2E*) and small ubiquitin-related modifier 1 (*SUMO-1*) genes. No expression of these genes was observed in the renal tubular epithelium of normal mice. However, proteins encoded by the *POLR2E* and *SUMO-1* genes were found in the renal tubular cells of mice with acute kidney injury after administration of MVs. No proteins were found in cells of animals that did not receive MVs [121]. An *in vitro* experiment demonstrated that MV genes were found in renal tubular cells after co-incubating them with MVs for 1 h.

A number of studies have demonstrated the ability of MVs to reduce the degree of apoptosis. Herrera *et al* [125]. used TUNEL staining to show that MVs derived from human hepatic stromal cells significantly reduced apoptosis after hepatectomy and enhanced cell regeneration. Treatment with RNase neutralized this effect, which confirmed the role of mRNAs and microRNAs in this process. It was shown in the model of cisplatin-induced renal failure that treatment with MVs increased expression of antiapoptotic genes (*BCL-XL*, *BCL2*, and *BIRC8* (baculoviral inhibitor of apoptosis repeat containing protein 8)), while reducing expression of pro-apoptotic agents such as caspase 1, caspase 8, and lymphotoxin-α. Moreover, introduction of MVs significantly increased PCNA expression by tubular cells, attesting to the important effect of MVs on proliferation. Micro- and mRNAs clearly play the key role in this effect [121].

Zhu *et al.* demonstrated that MVs isolated from human bone-marrow-derived MSCs alleviated *E. coli* endotoxin-induced acute lung injury, reduced the amount of extravascular fluid in lungs by 43%, and reduced the total protein level in broncho-alveolar fluid by 35%. Introduction of MVs also inhibited neutrophil migration and reduced MIP-2 concentration by 49%. Additionally, KGF gene knockout by RNA interference profoundly reduced the effect of MVs. We believe that the transfer of KGF mRNAs by MVs plays a key role in the effect exerted by MVs, MSCs and MSC-CM in endotoxin-induced acute lung injury [135]. There is no doubt that production of MVs is one of the key mechanisms in the paracrine effect of MSCs, along with secretion of soluble growth factors and cytokines. It is probably the synergistic activity of these two sets of agents that provides the powerful positive effect that has been repeatedly observed when using MSCs-CM to treat a number of different disease models.

## 35.3 Mesenchymal-stromal cells in the treatment of acute ischemia–reperfusion injury

IRI is a pathological process in which cell injury of an ischemic organ is enhanced by the restoration of blood flow to it [136–138].

Girn *et al* [139]. proposed the following definition of IRI: "the paradoxical and complex phenomenon of exacerbation of cellular dysfunction and increase in cell death after the restoration of blood flow to previously ischemic tissues."

Organ or tissue injuries such as acute myocardial infarction, acute renal failure, ischemic stroke, and tissue damage due to shock (severe hypotension) are the main fatal conditions that are accompanied by IRI.

Current treatment regimens of IRI are not fully effective, and new approaches to IRI therapy are needed. The use of MSCs is one approach, but first we would like to provide an overview of the pathogenesis of IRI.

### 35.3.1 Ischemia–reperfusion injury pathogenesis

The processes underlying the reperfusion syndrome can be defined as follows:

1 A metabolic imbalance accompanied by damage to mitochondria and disturbance of calcium homeostasis
2 Increased production of reactive oxygen species and NO.
3 Disorders of the microcirculation.
4 Activation cells of the immune system with the subsequent release of proinflammatory and pro-apoptotic factors.

Inflammatory changes in the form of the increased secretion of proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, and others by the ischemic tissue as well as the activation of complement components (C5a) leads to the development of inflammation and the migration of leukocytes, primarily neutrophils, to the damaged area [140]. Neutrophils activate adhesion molecules and interact with the endothelium through the rolling, adhesion, diapedesis mechanism [141], which, in combination with an increase in the rigidity of their membrane and a consequent decrease in their ability to deform, leads to disruption of blood flow throughout the capillary bed and prolongation of ischemia during the acute reperfusion stage – the no-flow phenomenon [142]. Of the proinflammatory cytokines, TNF-α is of particular importance, since it has been demonstrated to be capable of inducing heart microvascular endothelial dysfunction in the myocardium independently of neutrophil infiltration [143]. Furthermore, the action of anti-TNF antibodies results in a significant reduction in coronary arteriolar endothelial dysfunction and in the size of the myocardial infarct [140]. A direct relationship between the level of TNF-α expression and endothelial dysfunction during reperfusion injury has been observed [144].

The release of reactive oxygen species and free radicals (in particular, those due to increasing lipid peroxidation) leads in turn to elevated transaminase levels in the blood, increased inflammatory infiltrate, chromatin fragmentation, and the development of apoptosis [145].

Metabolic disorders are associated with an excessive intake of electrolytes and tissue macroerg synthesis products into damaged and necrotic tissues. Damaged tissues are not able to metabolize the incoming substrate normally. This leads to disruption of cell excitability, the activation of cyclogenase and lipoxygenase with subsequent formation of prostaglandins and leukotrienes, and the activation of anaerobic metabolism, which in turn exacerbates the degree of metabolic acidosis [146]. These changes lead to pronounced changes in cells, with apoptosis and necrosis of cells during acute reperfusion.

### 35.3.2 The use of mesenchymal stromal cells in kidney ischemia–reperfusion injury

Kidney IRI is the most frequent cause of IRIs, leading to acute renal failure in both the native kidney and the transplanted kidney [147]. The beneficial effects of MSCs in acute renal insufficiency (in particular, cisplatin-induced kidney failure) have been demonstrated in both *in vivo* and *in vitro* models. Administration of MSCs produced a significant increase in cell viability *in vitro* [58], the degree of apoptosis level was decreased [68], animal survival in *in vivo* experiments was increased up to 80% compared with control animals [52,148], and the function of the damaged organs was largely retained [52,53,60].

Chen *et al.* modeled kidney IRI in rats by clamping the renal artery pedicles on both sides for 1 h [88]. Next, the experimental group was administered adipose-derived MSCs (ADMSCs) while the control group received the culture medium. The urea and creatinine parameters at different intervals after reperfusion were examined. A significant improvement in the levels of both parameters was observed in the experimental group compared to the control group [88]. Histological studies revealed more significant damage to the kidney tissue in the control group compared with the experimental group, which was demonstrated by large areas of tubular necrosis, glomerular dilatation, and the loss of brush border.

Further, a study of mRNA expression of endothelin-1 (an indicator of vasoconstriction and endothelial damage), and TNF-α, and MMP-9 (indicators of inflammation) was conducted using real-time polymerase chain reaction. The values of these parameters in the control group were significantly higher than those in the experimental one, whereas anti-inflammatory markers such as IL-10 were significantly higher in the experimental group compared with the control. In addition, the secretion of ICAM-1 adhesion molecules and NF-κB were investigated using Western blot. These markers were also significantly reduced in the experimental group compared with the control group. In addition, the expression of markers of oxidative stress and apoptosis was investigated: an apoptosis indicator, caspase-3, was significantly reduced, whereas an antiapoptotic indicator, BCL-2, was increased in the experimental group compared with the control group.

Chinese researchers also demonstrated a pronounced positive effect of MSCs in a kidney IRI model *in vivo* and *in vitro* [147]. They focused on the increased release of SDF-1 (or CXCL12) by the ischemic kidney tissue. This factor plays a crucial role in the chemotaxis and homing of MSCs to inflamed tissue. The interaction of SDF-1 with MSCs is mediated by two chemokine receptors: CXCR4 and CXCR7. These researchers found that the expression of SDF-1 receptors was significantly increased by culturing MSCs under hypoxic (3% $O_2$) conditions. They found that CXCR4 was required for the homing of MSCs to the ischemic tissue. CXCR7 was important for increasing the viability of MSCs in the ischemic tissue.

Considering hydrogen peroxide ($H_2O_2$) as one of the most important mediators of cell death in IRI, Sachse and Wolf treated MSCs with this agent [149]. MSCs cultured in hypoxic conditions had much higher viability than MSCs cultured in normoxic conditions. As indicated above, a relationship and the expression of CXCR4 and CXCR7 receptors has been described. Four major pro-regenerative factors secreted by MSCs are VEGF,

HGF, IGF-1, and bFGF [52,68,74]. Using enzyme-linked immunosorbent assay, it was shown that the secretion of these growth factors was increased under hypoxic conditions and/or upon adding SDF-1 to the culture. At the same time, when CXCR4 and CXCR7 were neutralized by monoclonal antibodies, the secretion of all growth factors studied was markedly decreased. Furthermore, the therapeutic effect of MSCs in an *in vivo* kidney IRI model was diminished.

This work demonstrated the ability of MSCs not only to adapt well to conditions of hypoxia and ischemia, but also to increase their functional activity, including protein secretion, in a renal model of IRI due to the expression of SDF-1 and its receptors.

Another group conducted a similar study, but, in contrast to previous studies, they used uncultured ADMSCs freshly isolated from the adipose tissue of rats or fresh ADMSCs that had been cryopreserved [150]. The intra-arterial administration of these cells into the IRI-affected kidney increased the survival rate of the experimental animals and reduced the serum level of creatinine and urea compared with the control group within the first 24 h. Additionally, it also significantly reduced the expression of the proinflammatory cytokines IL-6 and MIP-2α (CXCL2). Furthermore, a significant decrease in $CD68^+$ macrophage infiltration of the kidney tissue was observed, as well as an increase in the cell proliferation marker Ki-67 in the distal and proximal kidney tubules after the administration of both freshly isolated ADMSCs and after ADMSCs that had been previously cryopreserved [150].

Since these changes were detected within the first day of IRI, as well as the fact that ADMSCs were virtually absent in the kidney tissue 72 h after the introduction, it is likely that the therapeutic effect in ischemia–reperfusion kidney injury is mediated by paracrine factors secreted by the ADMSCs and not by transdifferentiation [150].

### 35.3.3 Mesenchymal stromal cells and myocardial ischemia–perfusion injury

Acute coronary syndrome is a form of coronary heart disease that is the leading cause of mortality in the developed world. Timely reperfusion is the most important treatment. However, reperfusion itself is accompanied by inflammatory changes, oxidative stress, and metabolic disorders that lead to further damage to the myocardium. MSC administration into the myocardium has been reported to significantly reduce pathological remodeling of the infarct area (to become fibrotic), and thus to improve ventricular function [151–153]. MSC administration significantly reduced infarct size and increased cell proliferation and neovascularization of the infarcted area compared with hematopoietic progenitor cells [154]. A minority of investigators have explained this positive MSC effect by their ability to differentiate into cardiomyocytes *in situ* [151,153]. However, this remains controversial [155] and has not been able to be confirmed by other groups.

In contrast, there are a number of studies that suggest that paracrine mechanisms are involved in this improvement in myocardial function [154,156–161]. For example, Angoulvant *et al.* demonstrated that MSC-CM significantly reduced myocardial IRI. The *in vitro* data were obtained using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test and by measuring lactic dehydrogenase. The *in vivo* data were obtained by measuring blood levels of creatine phosphokinase and by measuring infarct area [162]. The MTT assay is a colorimetric assay for assessing cell viability: nicotinamide adenine dinucleotide phosphate (NADPH)-/reduced form of nicotinamide adenine dinucleotide-dependent cellular oxidoreductase enzymes may, under defined conditions, reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT to its insoluble formazan, which has a purple color. Moreover, the main mechanism of the positive effect was attributed in this study to paracrine activation of the PI3K-dependent pathway.

In 2006, Gnecchi *et al.* demonstrated that the introduction of CM obtained from MSCs overexpressing the Akt gene into the myocardium considerably reduced infarct area and improved left ventricular systolic pressure [156]. It was shown *in vitro* that this MSC-CM enhanced the viability of cardiomyocytes cultured under hypoxic conditions. As an indicator of apoptosis, the caspase-3 level was markedly reduced upon the introduction of the CM from MSCs. Additionally, the levels of paracrine factors such as VEGF, HGF, IGF-1, and bFGF were quite high in the CM of unmanipulated MSCs grown under normoxic conditions, but lower than in the CM from cells cultured under hypoxic conditions and/or those with enhanced expression of the Akt gene [156].

In another study, American investigators demonstrated that the MSC cardioprotective effect increased with knockout of the *TLR4* gene [55]. TLR4 is known to be expressed by MSCs [163]. When interacting with its ligand, this receptor is capable of inducing apoptosis and reducing cellular proliferative capacity, while a deficiency in this receptor leads to a decrease in the secretion of proinflammatory cytokines by cells [163,164]. Using the isolated rat heart, an IRI model was developed and parameters including left ventricular diastolic pressure and end-diastolic pressure were measured. TLR4 knockout (TLR4KO) murine MSCs demonstrated a more pronounced cardioprotective effect.

It has also been shown that TLR4KO MSCs produce more growth factors and chemokines – such as tissue inhibitor of metalloproteinases-1, M-CSF, and factors encoded by the KC and JE genes – and enhance MSC proliferation to a greater extent than wild-type MSCs [55].

In addition, it was noted that the baseline level of signal transducer and activator of transcription-3 (STAT3) was significantly higher in TLR4KO MSCs than in wild-type MSCs, and that knockdown of STAT3 using RNA interference caused a significant decrease in the production of growth factors and chemokines by TLR4KO MSCs compared with TLR4KO MSCs without STAT3 knockdown. The cardioprotective effect upon STAT3 knockdown was also decreased. Thus, it has been suggested that the enhanced production of cytokines and the cardioprotective effect of TLR4KO MSCs occurred through the STAT3-dependent pathway [55].

In another interesting study, Webber *et al.* developed a gel composed of self-assembling nanofibers of glycosaminoglycans linked to heparin-binding peptide amphiphile (HBPA) [165]. This gel is capable of immobilizing and binding to heparin, which makes it possible to bind various proteins through their heparin-binding domains. For example, VEGF and HGF bind to heparin, and hence to the gel. The objective of this study was to bind growth factors from MSC-CM and to test this on an IRI model of myocardial infarction and an IRI model of hind limb ischemia *in vivo*. Introduction of the gel steeped in the MSC CM into the wall of the left ventricle promoted significant retention of myocardial function, including left ventricular contractility, relaxation ability, and average systolic pressure, in a rat model of acute myocardial infarction compared with the untreated controls as well as with rats that received the separate introduction of the CM and the gel not steeped in the CM [165]. The most pronounced effect was obtained when the gel was steeped in CM from MSCs cultured under hypoxic (5% $O_2$) conditions. Similar results were obtained in a hind limb ischemia model. In this model, angiogenesis was assessed using angiography [165].

### 35.3.4 Mesenchymal stromal cells and ischemia–reperfusion injury of other organs

There have also been attempts to use MSCs in modeling IRI of other organs. For example, Kanazawa *et al.* demonstrated in a rat model of ischemia–reperfusion liver injury that transplantation of MSCs immediately after the restoration of perfusion significantly reduced the level of hepatic transaminases (a rise above normal indicates hepatic damage) and the level of apoptosis. Apoptosis was observed mainly in the centrilobular area. Enhancement of liver regeneration was noted [62]. Most of the introduced MSCs were in the periportal area, and only a small fraction of the cells was found in the centrilobular area. Thus, the cell migration site and the apoptosis site did not coincide, indicating that the transplanted cells did not replace damaged hepatocytes and that the antiapoptotic effect was likely due to paracrine mechanisms. In addition, the presence of the MSCs was determined by their luciferase activity of cells using an *in vivo* imaging system (IVISTM; Xenogen, Alameda, CA). The number of MSCs significantly decreased with time, and virtually none were observed after 7 days [62]. The significant reduction in liver transaminases in the first 24 h after MSC administration suggests that their effect was mediated by paracrine mechanisms [62].

The administration of MSCs as treatment of ischemia–reperfusion lung injury has also been described in a number of studies [166–168]. Sun *et al.* demonstrated in a rat model of lung IRI that administration of ADMSCs markedly increased the $O_2$ saturation level in blood and lowered the systolic blood pressure in the right ventricle as early as 72 h after the start of reperfusion compared with the control group, indicating protection of lung function. They found a significant increase in the level of expression and secretion of proinflammatory cytokines, including TNF-α, IL-1β, MMP-9, NF-κB, VCAM-1 and ICAM-1, pro-apoptotic factors (caspase-3, BAX), and endothelin-1 in the control group compared with the experimental group. In contrast, the experimental group showed enhanced expression of anti-inflammatory agents (including adiponectin, endothelial NO synthetase, and IL-10), as well as the anti-apoptotic agent BCL-2 and the antioxidant factors heme oxygenase, NADPH oxidase, NADPH quinone reductase, and glutathione reductase. Additionally, there was enhanced expression in the experimental group of von Willebrand factor and $CD31^+$, indicating active angiogenesis [166]. Chen *et al.* obtained similar results, although they used MSCs derived from bone marrow rather than from adipose tissue [167].

In another study, bone-marrow-derived MSCs transduced with the IL-10 gene were used for treatment of lung IRI *in vivo*. There was a marked improvement in both structural and functional parameters of the lungs in the experimental group compared with an untreated control group and a group given unmodified MSCs. Parameters of inflammation and apoptosis were also significantly reduced in the experimental group [168].

Other studies have reported positive outcomes when MSCs were administered in preclinical models of IRI of the intestine [169], the retina [170], the brain [171], and the limbs [58].

### 35.3.5 Conclusions

MSCs appear to have a beneficial effect in a number of organs damaged by IRI, and this effect appears to be mediated by a paracrine function of the MSCs and not by transdifferentiation.

## 35.4 The use of mesenchymal stromal cells in acute lung and airway injury

Pulmonary epithelium is constantly exposed to pathogenic factors such as microorganisms, allergens, smoke, and occasionally to chemical gases. Physiologic repair and renewal of airway epithelial cells is a continuous process, although its rate is lower than that of renewal of skin or colonic epithelium.

Repair of airway epithelium after an acute injury depends on the intensity of a damaging factor, on the type of pathogenic agent, and the injured area, be it the trachea, the bronchi, or the alveoli.

The epithelium of mouse trachea consists of a number of different cell types. Ciliary cells express β-tubulin and forkhead protein J1 (FOXJ1). Club cells, also known as bronchoalveolar cells and originally known as Clara cells after their discoverer Max Clara (1899–1966), express secretoglobin family 1A member 1 (SCGB1A1, also known as uteroglobin) and mucin 5b. Goblet cells express Scg1a1, Muc5B, Muc5A/C, and SAM pointed domain-containing ETS transcription factor. Basal cells express transformation-related protein 63 (p63) and cytokeratin 5 and/or 14 [172–177].

The mucus-secreting glands of the tracheal mucosa are located between the epithelial layer and the cartilage tissue. These glands have been shown to contain ductal cells that express cytokeratin 5

and 14 and, together with basal cells, are involved in the regeneration of severe epithelial injury that can be caused by ischemia or tracheal transplantation [178,179].

Columnar ciliated epithelium is replaced by monolayer cubic epithelium closer to the distal regions of the lungs. The intralobular bronchi mostly contain club, ciliary, and neuroendocrine cells. The highest concentration of neuroendocrine cells is at the bronchial bifurcation zone [177].

In the distal regions of the lung, type I pneumocyte cells (also called type I alveolar cells or squamous alveolar cells) are extremely attenuated cells that line the alveolar surfaces of the lungs. They cover 97% of the alveolar surface, with type II pneumocytes covering the remainder. Type II pneumocytes, also called alveolar type II cells, great alveolar cells, or septal cells, are granular and roughly cuboidal in shape. Type II pneumocytes are typically found at the alveolar-septal junction. Although they comprise 60% of the alveolar lining cells, because of their shape they cover a much smaller surface area than type I cells (<5%). Type II pneumocytes secrete a surfactant that is composed of a group of phospholipids that reduce alveolar surface tension.

### 35.4.1 Repair of the proximal regions of the airways after acute injury

Chemical agents such as sulphur dioxide, hydrochloric acid and naphthalene damage the ciliary epithelium. Club cells and basal cells expressing p63 and keratin type II cytoskeletal 5 (KRT5) that are involved in repair are partially retained. Ciliary cells and goblet cells recover during the repair process [174,180–183]. Diphtheria toxin mostly kills basal cells, and the surviving club cells are involved in their regeneration. Ductal stromal cells expressing Krt5 and Krt14, which reside in submucosal glands and which repair all types of epithelial cells, are activated in severe tracheal injury caused by hypoxia or transplantation, when almost all epithelial cells are damaged [178,179].

Data on the repair of the distal bronchial regions were obtained using the naphthalene-induced injury model. Naphthalene injury selectively kills club cells in intralobular bronchi, while the pool of naphthalene-resistant club cells (known as variant club cells) is retained. The latter cells express SCGB1A1 and surfactant protein C and reside near neuroendocrine cells and the bronchoalveolar junction. Neuroendocrine cells express calcitonin gene related peptide and protein gene product 9.5. The surviving variant club cells begin to secrete Wnt7b, which induces active proliferation of airway smooth muscle cells. The cells secrete FGF10, which induces short-term epithelial to mesenchymal transition. As a result, club cells begin to proliferate and repair the injured sites. Inhibition of FGF10 significantly reduces the ability of bronchial epithelium to regenerate after naphthalene-induced injury. It should be mentioned that neuroendocrine cells can be transformed into club cells. However, their contribution to the repair process is insignificant, since their elimination does not have a noticeable effect on regeneration [177].

In addition to resident stromal cells in the lung tissue, the mouse bone marrow contains $KRT5^+$ cells that participate in the repair of tracheal epithelium. It has been demonstrated that $CD45^+$ $KRT5^+$ cells expressing CXCR4 maintained the circulating pool involved in epithelium repair after injury. The cells are mobilized by activation of the CXCL12/CXCR4 axis. $KRT5^+$ $CXCR4^+$ cells play a key role in tracheal graft re-epithelization, and blocking of CXCL12 prevents mobilization of these cells [184].

It has also been demonstrated that bone-marrow-derived endothelial progenitor cells express KGF receptor in addition to $KRT5^+$. Systemic administration of KGF significantly increased mobilization of circulating endothelial progenitor cells 6 h after injection. A more rapid repair was observed in mice with transplanted trachea that received KGF due to the increased number of circulating endothelial progenitor cells. This effect was inhibited by anti-CXCL12 antibodies [185].

The role of MSCs in the repair of tracheal epithelium has been shown in many studies. Gender mismatch (detection of the Y chromosome) or green fluorescent protein was used to demonstrate that bone-marrow-derived MSCs contributed to the repair of epithelium in the proximal and distal airways regions. However, the number of MSCs in the lung tissue was low, ranging from 1 to 14% [186,187].

The intratracheal administration of MSCs in a mouse model of LPS-induced lung injury showed that the transplanted cells reduce the TNF and MIP-2 levels, while increasing the IL-10 level in broncho-alveolar lavage fluid. The degree of pulmonary edema was decreased and survival increased from 42% to 80%. Although less than 5% of MSCs were detected in lung tissue, intratracheal administration of MSCs had a therapeutic effect [187]. Similar data were obtained with the intravenous injection of MSCs in a bleomycin-induced model of respiratory failure [188,189]. Bleomycin is a cytotoxic drug that can cause pulmonary fibrosis both in humans and in mice. Because of the low numbers of MSCs detectable in lung tissue, their therapeutic effect was likely mediated by a paracrine effect.

In 2014, a study showed that IL-6 stimulated regeneration of ciliary cells while STAT3 inhibitors reduced it. IL-6/STAT3 signaling facilitated activation of multiciliary genes and enhanced expression of the *FOXJ1* gene. Multiciliated cells are a specialized population of post-mitotic cells decorated with dozens of motile cilia that beat in a polarized and synchronized fashion to drive directed fluid flow across an epithelium. Suppression of the STAT3 signaling pathway increased the number of secretory and basal cells. IL-6 null mice with lung injury produced less ciliary cells and a greater number of secretory cells [190].

We studied the effect of MSC-CM plus MSC lysate (MSC-CM-L) on the repair of rat tracheal epithelium after burn injury resulting from steam inhalation. MSC-CM combined with rat MSC lysate has a marked regenerative effect in heat-induced burn of the tracheal epithelium. Twenty-four hours after heat-induced injury histological examination showed that the

epithelium in the upper portion of the trachea was damaged through to the submucous layer (Figure 35.5A and B).

In the middle portion of the trachea more than 50% of the mucous membrane was damaged. The integrity of the epithelial layer remained partially disrupted (Figure 35.5C).

The pathological changes observed included an expanded vascular network, increased permeability of blood vessels, red blood cells in the epithelial layer, and necrosis of a large number of columnar epithelial cells. There was intense inflammation in the epithelium. Goblet cells and mucous glands retained their secretory properties in the surviving regions of the mucosa.

In this preclinical burn model, the epithelium was partially repaired 2–3 weeks after the injury (Figure 35.5D).

Inhalation of MSC-CM-L after the burn increased the size of the submucosal layer and the structures it contained, such as the submucosal glands, as early as 3 days after the burn. The structures migrated toward the basement membrane to be incorporated into the tracheal epithelium (Figure 35.6A and B).

Analysis demonstrated that the resulting vesicles contained ciliary cells of respiratory epithelium (Figure 35.7).

The epithelial layer in the experimental group of animals was completely repaired by 5–7 days after the injury (Figure 35.6C).

The acceleration of tracheal epithelium repair in animals after inhalation injury combined with MSC-CM-L administration can be attributed to accelerated proliferation and differentiation of ductal stromal cells and to the protective effect of cytokines and growth factors contained in MSC-CM-L. We believe that a key aspect of the protective effect is the impact of VEGF, which exhibits both antiapoptotic and anti-inflammatory effects. Inhalation of IL-6 also played a role. When inhaled, this cytokine exhibited an anti-inflammatory effect and reduced the cytokine

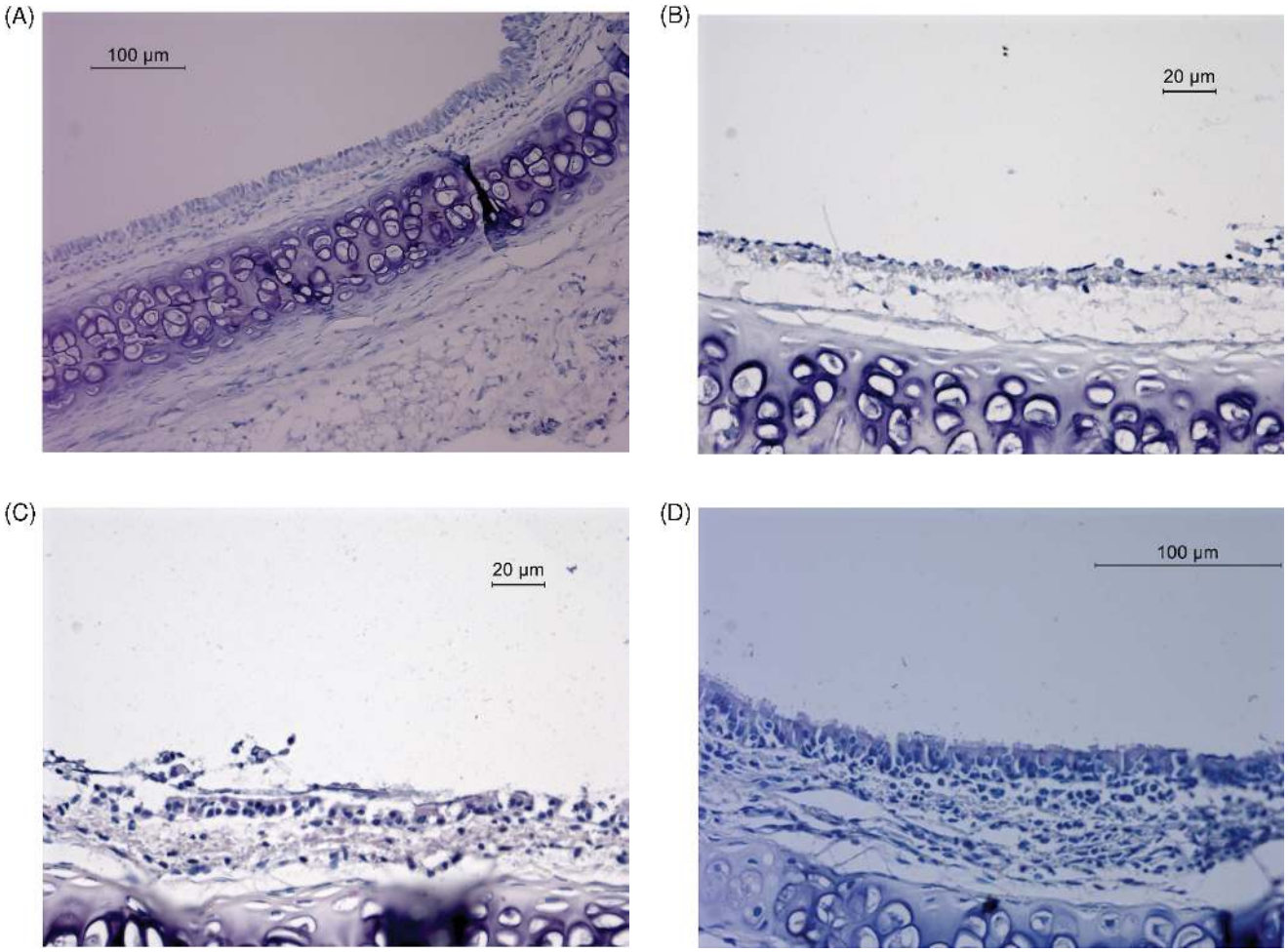



**Figure 35.5** Histopathologic findings in rat tracheal epithelium after a thermal burn:. (A) intact control; (B) 24 h after thermal burn; (C) 7 days after thermal burn; (D) 14 days after thermal burn. Mass epithelial cell death is observed following the burn (B); the epithelium is partly restored at 7 days (C), and complete or nearly complete epithelial recovery is seen at 14 days (D). Staining by haematoxylin–eosin.

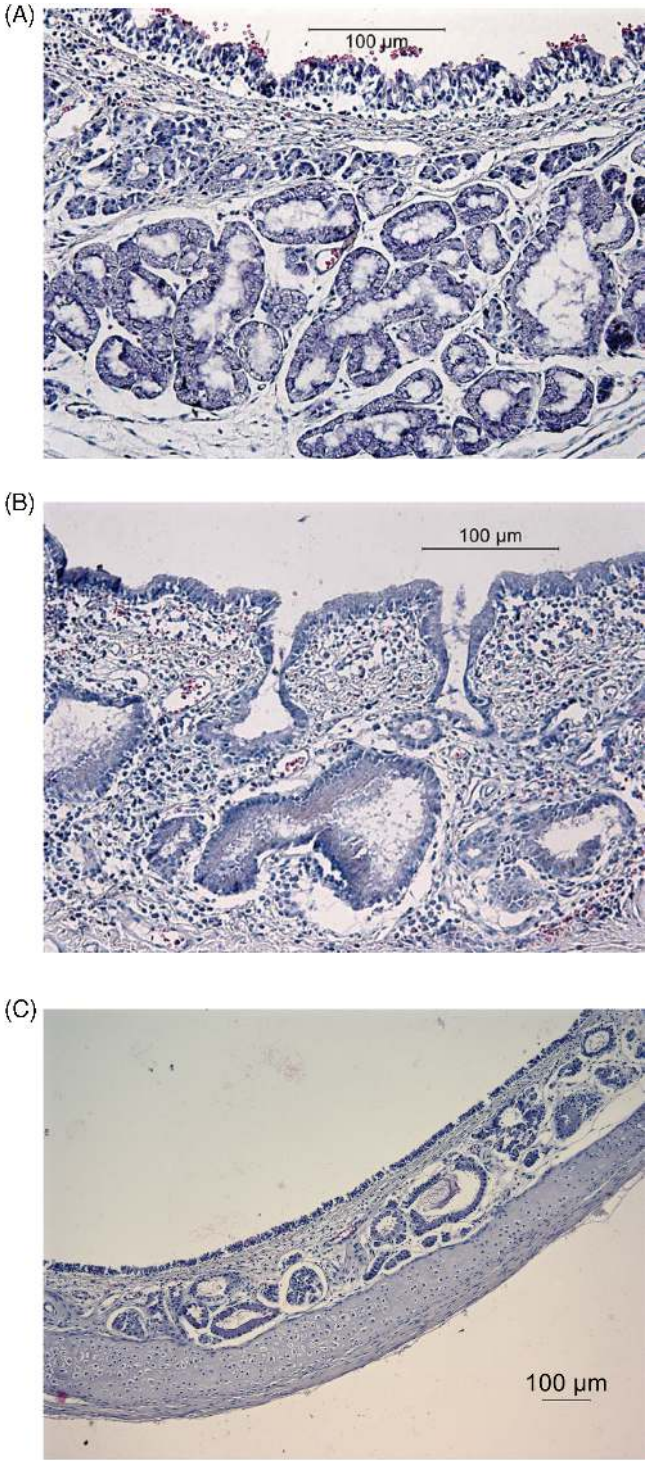



**Figure 35.6** Histopathologic findings in rat tracheal epithelium after thermal burn in combination with intratracheal administration of MSC-CM-L. Endotracheal introduction of MSC-CM-L after the burn resulted in vesicle formation in the submucosa of the trachea. (A, B) The greatest number of vesicles in the submucosa was observed on day 3 following the burn injury and MSC-CM-L administration. After this time point the vesicles reduced in number, reaching a minimum by day 7 (C). Moving toward the basal membrane, the vesicles became incorporated into the respiratory epithelium of the trachea (B). Staining by haematoxylin–eosin.

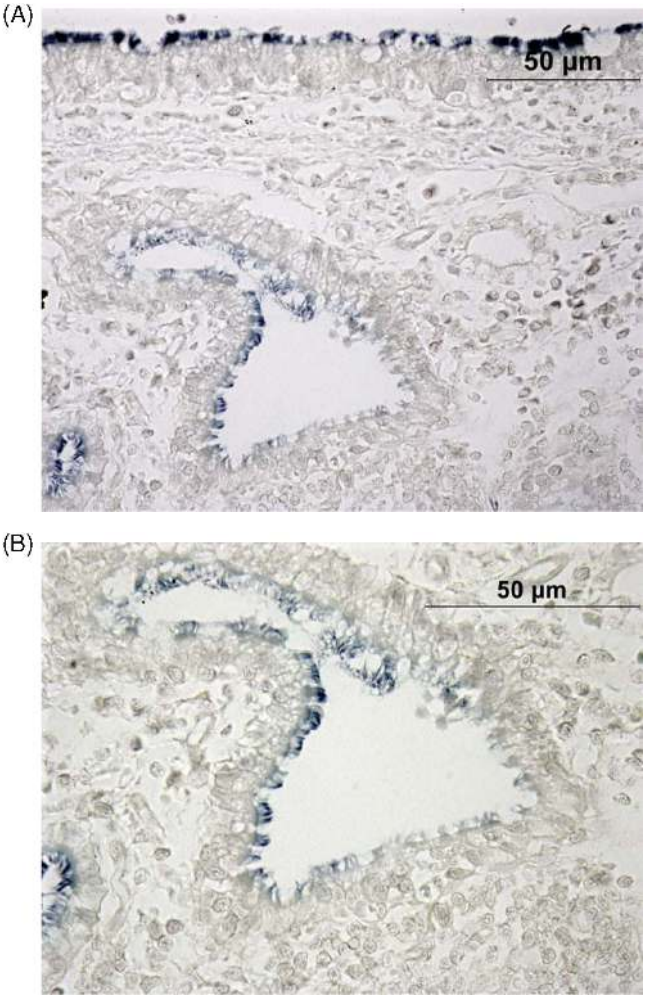



**Figure 35.7** Histopathologic findings in rat tracheal epithelium after a thermal burn in combination with intratracheal administration of MSC-CM-L. (A) Vesicles in the submucosa of the trachea after thermal burn at day 3 after MSC-CM-L administration. Staining by haematoxylin–eosin. (B) Immunohistochemical detection of ciliated cells of the epithelium. The primary antibody used was mouse monoclonal anti-tubulin; the secondary antibody was anti-mouse antibody conjugated with alkaline phosphatase.

level in inflamed lung caused by the intratracheal administration of LPS [98].

The IL-6 level in MSC-CM-L used was 1200 pg/mL. The increase in the IL-6 level in the tracheal lumen after heat-induced injury appeared to be a factor responsible for the predominant emergence of ciliary cells in the vesicles formed in the submucosal layer. This phenomenon might be caused by the effect of cytokines, as previously described by Tadokoro *et al* [190].

The findings demonstrate that paracrine mechanisms of MSCs play a crucial role in the repair of tracheal epithelium after acute injury. The acellular agent from stromal cells might be effective in treatment of acute lung injury in humans.

## 35.5 Current approaches to controlled transplantation of mesenchymal stromal cells in acute organ injury

The use of autologous MSCs is theoretically preferential to the use of allogeneic MSCs. Their transplantation does not induce immune rejection and minimizes the risks of viral contamination or reactivation. However, prolonged *ex vivo* cell culture increases the risk of malignant transformation and increases the risk of long-term complications [191,192]. In practical terms it is not possible to produce autologous MSCs soon after injury.

The limitations of using allogeneic MSCs also includes the risk of viral infection, although careful screening of the MSC donor similar to that applied to blood donors can minimize this risk. Transplantation of these cells, in particular in immune-compromised patients, could theoretically contribute to tumor development if the cells enter immune-privileged organs or may have a general negative effect on the immune status [193,194]. However, to date, the use of allogeneic MSCs appears to be safe.

Transplantation of MSCs exhibiting high secretory activity or MSCs transfected with genetic constructs and producing neurotrophic factors can be regarded as an alternative approach to therapy of a number of diseases, and retinal diseases in particular [195–204].

It is may be important to deliver nerve growth factor in neurodegenerative disorders of the eye. Successful transfection of human embryonic kidney (HEK) 293 cells with NGF has been reported: *in vitro* NGF expression from transfected HEK 293 cells was observed for 5 days [204] and up until 9 days [205].

As indicated earlier, one theoretical limitation of using MSC transplantation into immune-privileged organs (e.g., the eye or the central nervous system) is the potential risk of malignant transformation of the transplanted cells [206–209]. Again, no such events have been described of human MSCs in clinical trials.

There are a number of approaches to retain the transplanted cells within eye tissues; for example, the use of semipermeable capsules implanted into the vitreous body [210–212], which maintain diffusion of neurotrophic factors and prevent migration and uncontrolled proliferation of the transplanted cells, but may cause local inflammation and disrupt the translucency of the eye [212].

It is now possible to use magnetic technologies to anchor transplanted cells at the transplantation site. MSCs containing magnetic nanoparticles were used to deliver intravenously transplanted MSCs to the liver [213,214]. MSCs labeled with magnetic nanoparticles have also been delivered to the site of acute cardiovascular injury [215]. The directed and controlled migration of MSCs toward the injury site under an external magnetic field and local fixation of the material were attained during the observation period.

One disadvantage of such proposed methods is the high (up to 75%) percentage of MSC death within 24 h after their labeling with nanoparticles [215].

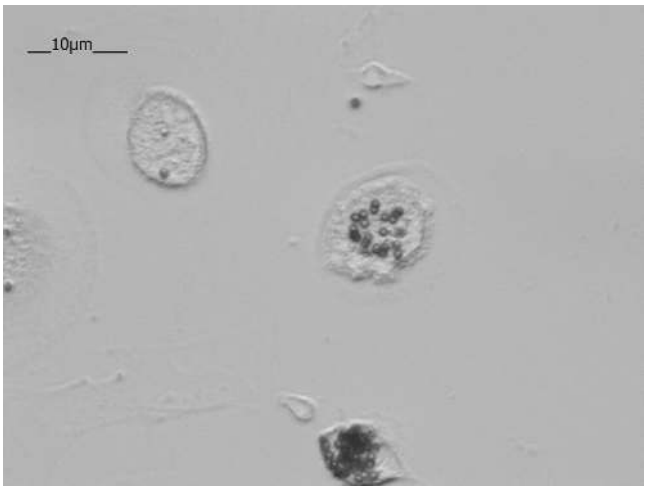



**Figure 35.8** MSC with magnetic particles. Using optical microscopy, different focal depths indicated that the magnetic particles were located in the cytoplasm.

In 2012, Yanai *et al.* successfully targeted delivery of MSCs labeled with superparamagnetic nanoparticles into the retina. However, the experimental duration was short and the study did not attempt to fix the transplanted cells at the transplantation site [216].

We have used micrometer-sized magnetic particles (Invitrogen) treated with Pluronic (a triblock copolymer) surfactants to produce MSCs containing micro-sized magnetic particles within their cytoplasm (Figure 35.8).

The transplanted cells were transfected with the enhanced green fluorescent protein gene using a Clontech kit for visualization. After a magnetic implant was added to a Petri dish, the cells were securely attached to its surface (Figure 35.9).

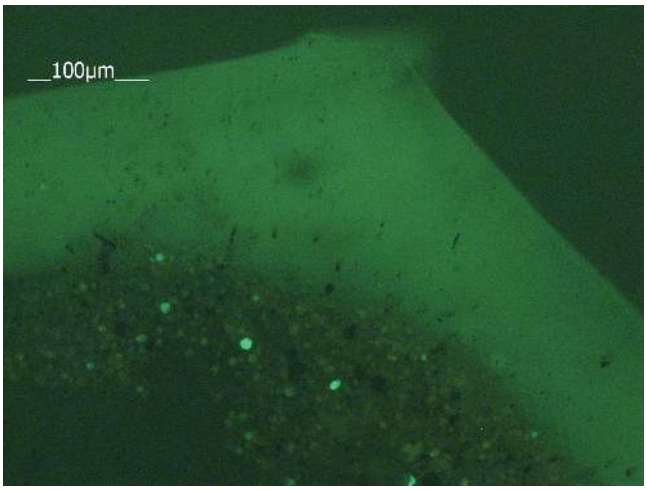



**Figure 35.9** Attachment of MSCs containing magnetic particles in their cytoplasm to the surface of a magnetic implant with a magnetic field intensity of 5 mT.

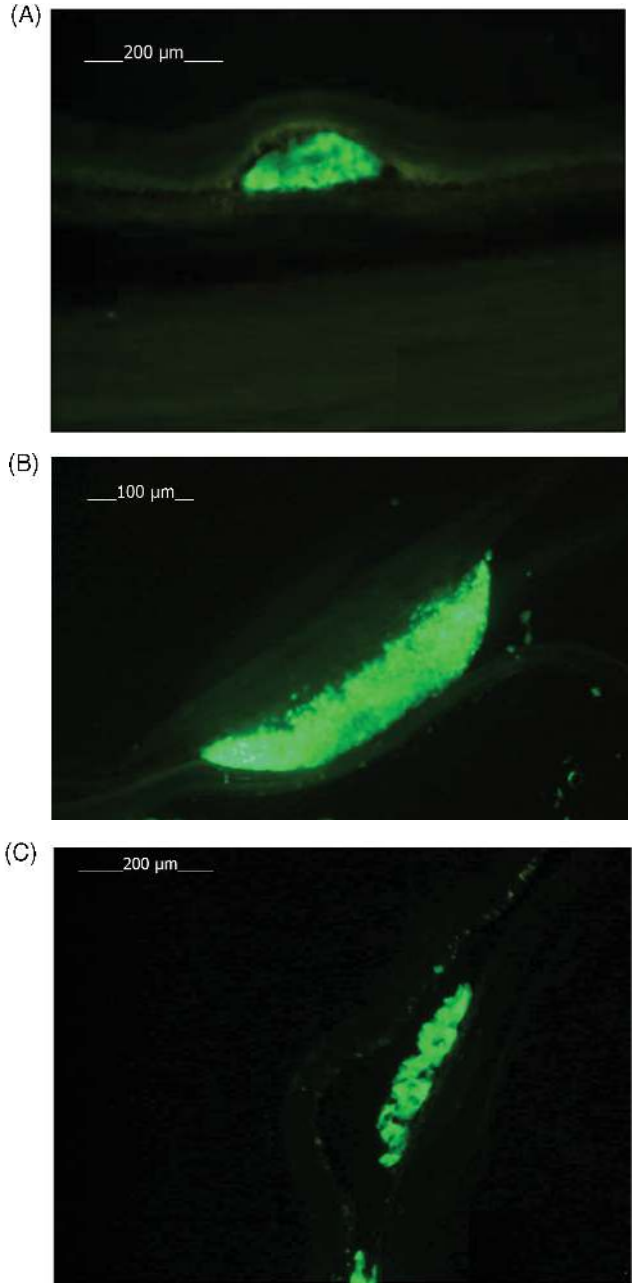



**Figure 35.10** MSCs with magnetic particles fixed by magnetic implant. The cells were located subretinally. Fluorescent microscopy was optimized for detection of green fluorescent protein. The transplantation site of the cells was fixed with a magnetic implant on day 5 (A), day 14 (B), and day 21 (C).

MSCs labeled with magnetic particles and retained by a magnetic implant were transplanted into the retina of rabbits. At 5, 14, and 21 days after the procedure the cells remained at the transplantation site (Figure 35.10). The cells could not be detected after 1 month.

When MSCs were transplanted into the retina without fixation using a magnetic implant, they started migrating to the vitreous body as soon as 3 days after transplantation and could not be detected in the retina by day 5 (Figure 35.11).

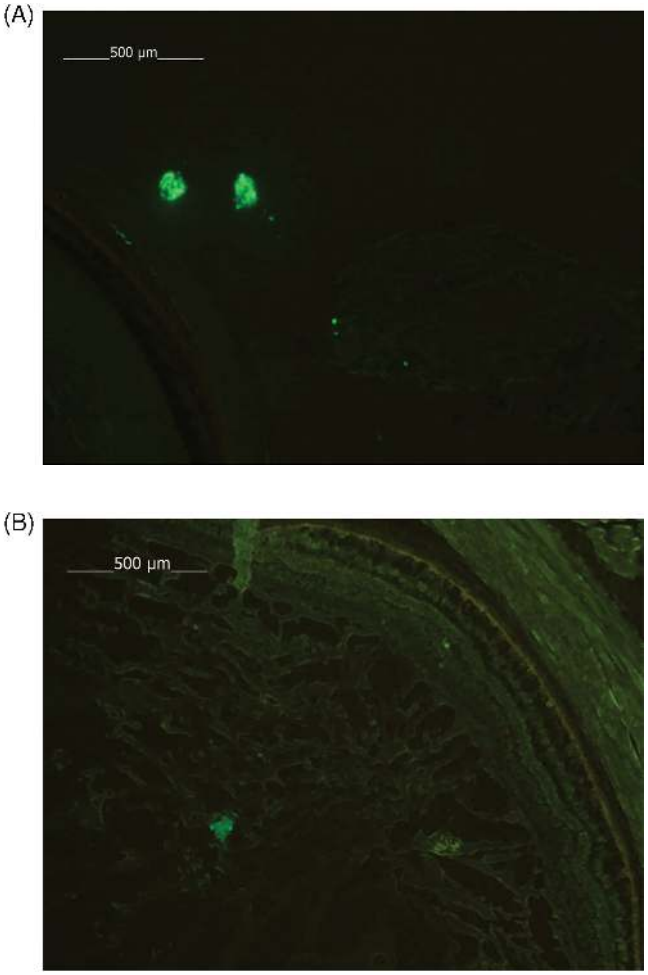



**Figure 35.11** Transplantation of MSCs with magnetic particles without a magnetic implant. The cells are located subretinally. Fluorescent microscopy was optimized for detection of green fluorescent protein. (A) The transplantation site of unfixed cells on day 3. Cells are located in the vitreous body cavity. (B) The transplantation site of unfixed cells on day 5.

These studies demonstrated that the proposed procedure ensured attachment of MSCs at the transplantation site for up to 21 days. This duration was seven times longer than that in the control group. This procedure opens up new avenues for studying the mechanisms of action of MSCs and designing new promising strategies for treating acute organ injury using allogeneic MSCs.

## 35.6 Conclusions

It is clear that the paracrine factors secreted by MSCs contribute to their protective and regenerative effects on acutely injured tissues and organs. Separation of their anti-inflammatory, immunomodulatory, regenerative, antiapoptotic, and antioxidant effects requires further elucidation. All these effects are interrelated and are often caused by similar processes.

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## References

1 Han J, Zou Z, Zhu C, *et al.* DNA synthesis of rat bone marrow mesenchymal stem cells through α1-adrenergic receptors. *Arch Biochem Biophys* 2009;**490**(2):96–102.
2 Fonseca RB, Mohr AM, Wang L, *et al.* The impact of a hypercatecholamine state on erythropoiesis following severe injury and the role of IL-6. *J Trauma* 2005;**59**(4):884–889.
3 Dasu MR, Ramirez SR, La TD, *et al.* Crosstalk between adrenergic and toll-like receptors in human mesenchymal stem cells and keratinocytes: a recipe for impaired wound healing. *Stem Cells Transl Med* 2014;**3**(6):745–759.
4 Csete M. Oxygen in the cultivation of stem cells. *Ann N Y Acad Sci* 2005;**1049**:1–8.
5 Ma T, Grayson WL, Fröhlich M, Vunjak-Novakovic G. Hypoxia and stem cell-based engineering of mesenchymal tissues. *Biotechnol Prog* 2009;**25**(1):32–42.
6 Grayson WL, Zhao F, Bunnell B, Ma T. Hypoxia enhances proliferation and tissue formation of human mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;**358**(3):948–953.
7 D'Ippolito G, Diabira S, Howard GA, *et al.* Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *J Cell Sci* 2004;**117**(Pt 14):2971–2981.
8 D'Ippolito G, Diabira S, Howard GA, *et al.* Low oxygen tension inhibits osteogenic differentiation and enhances stemness of human MIAMI cells. *Bone* 2006;**39**(3):513–522.
9 Berniakovich I, Giorgio M. Low oxygen tension maintains multipotency, whereas normoxia increases differentiation of mouse bone marrow stromal cells. *Int J Mol Sci* 2013;**14**(1):2119–2134.
10 Madrigal M, Rao KS, Riordan NH. A review of therapeutic effects of mesenchymal stem cell secretions and induction of secretory modification by different culture methods. *J Transl Med* 2014;**12**(1):260.
11 Lennon DP, Edmison JM, Caplan AI. Cultivation of rat marrow-derived mesenchymal stem cells in reduced oxygen tension: effects on *in vitro* and *in vivo* osteochondrogenesis. *J Cell Physiol* 2001;**187**(3):345–355.
12 Ren H, Cao Y, Zhao Q, *et al.* Proliferation and differentiation of bone marrow stromal cells under hypoxic conditions. *Biochem Biophys Res Commun* 2006;**347**(1):12–21.
13 Nagano M, Kimura K, Yamashita T, *et al.* Hypoxia responsive mesenchymal stem cells derived from human umbilical cord blood are effective for bone repair. *Stem Cells Dev* 2010;**19**(8):1195–1210.
14 Tsai CC, Yew TL, Yang DC, *et al.* Benefits of hypoxic culture on bone marrow multipotent stromal cells. *Am J Blood Res* 2012;**2**(3):148–159.
15 Toma C, Pittenger MF, Cahill KS, *et al.* Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 2002;**105**(1):93–98.
16 Mangi AA, Noiseux N, Kong D, *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 2003;**9**(9):1195–1201.
17 Zhu W, Chen J, Cong X, *et al.* Hypoxia and serum deprivation-induced apoptosis in mesenchymal stem cells. *Stem Cells* 2006;**24**(2):416–425.
18 Hu X, Yu SP, Fraser JL, *et al.* Transplantation of hypoxia-preconditioned mesenchymal stem cells improves infarcted heart function via enhanced survival of implanted cells and angiogenesis. *J Thorac Cardiovasc Surg* 2008;**135**(4):799–808.
19 Jin H, Sanberg PR, Henning RJ. Human umbilical cord blood mononuclear cell-conditioned media inhibits hypoxic-induced apoptosis in human coronary artery endothelial cells and cardiac myocytes by activation of the survival protein Akt. *Cell Transplant* 2013;**22**(9):1637–1650.
20 Maslov LN, Podoksenov IuK, Portnichenko AG, Naumova AV. Hypoxic preconditioning of stem cells as a new approach to increase the efficacy of cell therapy for myocardial infarction. *Vestn Ross Akad Med Nauk* 2013; (12):16–25 (in Russian).
21 Xiang MX, He AN, Wang JA, Gui C. Protective paracrine effect of mesenchymal stem cells on cardiomyocytes. *J Zhejiang Univ Sci B* 2009;**10**(8):619–624.
22 Lavrentieva A, Majore I, Kasper C, Hass R. Effects of hypoxic culture conditions on umbilical cord-derived human mesenchymal stem cells. *Cell Commun Signal* 2010;**8**:18.
23 Jaakkola P, Mole DR, Tian YM, *et al.* Targeting of HIF-α to the von Hippel–Lindau ubiquitylation complex by $O_2$-regulated prolyl hydroxylation. *Science* 2001;**292**(5516):468–472.
24 Lord-Dufour S, Copland IB, Levros Jr LC, *et al.* Evidence for transcriptional regulation of the glucose-6-phosphate transporter by HIF-1α: targeting G6PT with mumbaistatin analogs in hypoxic mesenchymal stromal cells. *Stem Cells* 2009;**27**(3):489–497.
25 Martin-Rendon E, Hale SJ, Ryan D, *et al.* Transcriptional profiling of human cord blood $CD133^+$ and cultured bone marrow mesenchymal stem cells in response to hypoxia. *Stem Cells* 2007;**25**(4):1003–1012.
26 Rosová I, Dao M, Capoccia B, *et al.* Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells* 2008;**26**(8):2173–2182.
27 Hung SC, Pochampally RR, Hsu SC, *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* 2007;**2**(5):e416.
28 Ahluwalia A, Tarnawski AS. Critical role of hypoxia sensor – HIF-1α in VEGF gene activation. Implications for angiogenesis and tissue injury healing. *Curr Med Chem* 2012;**19**(1):90–97.
29 Imtiyaz HZ, Simon MC. Hypoxia-inducible factors as essential regulators of inflammation. *Curr Top Microbiol Immunol* 2010;**345**:105–120.
30 Ohnishi S, Yasuda T, Kitamura S, Nagaya N. Effect of hypoxia on gene expression of bone marrow-derived mesenchymal stem cells and mononuclear cells. *Stem Cells* 2007;**25**(5):1166–1177.
31 Potier E, Ferreira E, Andriamanalijaona R, *et al.* Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression. *Bone* 2007;**40**(4):1078–1087.
32 Crisostomo PR, Markel TA, Wang Y, Meldrum DR. Surgically relevant aspects of stem cell paracrine effects. *Surgery* 2008;**143**(5):577–581.

33 Wang M, Crisostomo PR, Herring C, *et al.* Human progenitor cells from bone marrow or adipose tissue produce VEGF, HGF, and IGF-I in response to TNF by a p38 MAPK-dependent mechanism. *Am J Physiol Regul Integr Comp Physiol* 2006;**291**(4):R880–R884.

34 Muir C, Chung LW, Carson DD, Farach-Carson MC. Hypoxia increases VEGF-A production by prostate cancer and bone marrow stromal cells and initiates paracrine activation of bone marrow endothelial cells. *Clin Exp Metastasis* 2006;**23**(1):75–86.

35 Wu EH, Li HS, Zhao T, *et al.* Effect of hypoxia on the gene profile of human bone marrow-derived mesenchymal stem cells. *Sheng Li Xue Bao* 2007;**59**(2):227–232 (in Chinese).

36 Chang CP, Chio CC, Cheong CU, *et al.* Hypoxic preconditioning enhances the therapeutic potential of the secretome from cultured human mesenchymal stem cells in experimental traumatic brain injury. *Clin Sci (Lond)* 2013;**124**(3):165–176.

37 Yu J, Yin S, Zhang W, *et al.* Hypoxia preconditioned bone marrow mesenchymal stem cells promoted liver regeneration in a rat massive hepatectomy model. *Stem Cell Res Ther* 2013;**4**(4):83.

38 Li JH, Zhang N, Wang JA. Improved anti-apoptotic and anti-remodeling potency of bone marrow mesenchymal stem cells by anoxic pre-conditioning in diabetic cardiomyopathy. *Endocrinol Invest* 2008;**31**(2):103–110.

39 Park BS, Kim WS, Choi JS, *et al.* Hair growth stimulated by conditioned medium of adipose-derived stem cells is enhanced by hypoxia: evidence of increased growth factor secretion. *Biomed Res* 2010;**31**(1):27–34.

40 Grayson WL, Zhao F, Izadpanah R, *et al.* Effects of hypoxia on human mesenchymal stem cell expansion and plasticity in 3D constructs. *J Cell Physiol* 2006;**207**(2):331–339.

41 Choi KS, Shin JS, Lee JJ, *et al. In vitro* trans-differentiation of rat mesenchymal cells into insulin-producing cells by rat pancreatic extract. *Biochem Biophys Res Commun* 2005;**330**(4):1299–1305.

42 Chang SA, Lee EJ, Kang HJ, *et al.* Impact of myocardial infarct proteins and oscillating pressure on the differentiation of mesenchymal stem cells: effect of acute myocardial infarction on stem cell differentiation. *Stem Cells* 2008;**26**(7):1901–1912.

43 Labovsky V, Hofer EL, Feldman L, *et al.* Cardiomyogenic differentiation of human bone marrow mesenchymal cells: role of cardiac extract from neonatal rat cardiomyocytes. *Differentiation* 2010;**79**(2):93–101.

44 Schittini AV, Celedon PF, Stimamiglio MA, *et al.* Human cardiac explant-conditioned medium: soluble factors and cardiomyogenic effect on mesenchymal stem cells. *Exp Biol Med (Maywood)* 2010;**235**(8):1015–1024.

45 Bentz K, Molcanyi M, Schneider A, *et al.* Extract derived from rat brains in the acute phase following traumatic brain injury impairs survival of undifferentiated stem cells and induces rapid differentiation of surviving cells. *Cell Physiol Biochem* 2010;**26**(6):821–830.

46 Rigamonti E, Zordan P, Sciorati C, *et al.* Macrophage plasticity in skeletal muscle repair. *Biomed Res Int* 2014;**2014**:560629.

47 Ionescu L, Byrne RN, van Haaften T, *et al.* Stem cell conditioned medium improves acute lung injury in mice: *in vivo* evidence for stem cell paracrine action. *Am J Physiol Lung Cell Mol Physiol* 2012;**303**(11):L967–L977.

48 Bosurgi L, Corna G, Vezzoli M, *et al.* Transplanted mesoangioblasts require macrophage IL-10 for survival in a mouse model of muscle injury. *J Immunol* 2012;**188**(12):6267–6277.

49 Shohara R, Yamamoto A, Takikawa S, *et al.* Mesenchymal stromal cells of human umbilical cord Wharton's jelly accelerate wound healing by paracrine mechanisms. *Cytotherapy* 2012;**14**(10):1171–1181.

50 Friedenstein AJ, Petrakova KV, Kurolesova AI, Frolova, GP. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 1968;**6**:230–247.

51 Haynesworth SE, Baber MA, Caplan AI. Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1α. *J Cell Physiol* 1996;**166**(3):585–592.

52 Morigi M, Rota C, Montemurro T, *et al.* Life-sparing effect of human cord blood-mesenchymal stem cells in experimental acute kidney injury. *Stem Cells* 2010;**28**(3):513–522.

53 Morigi M, Introna M, Imberti B, *et al.* Human bone marrow mesenchymal stromal cells accelerate recovery of acute renal injury and prolong survival in mice. *Stem Cells* 2008;**26**(8):2075–2082.

54 Bi B, Schmitt R, Israilova M, *et al.* Stromal cells protect against acute tubular injury via an endocrine effect. *J Am Soc Nephrol* 2007;**18**:2486–2496.

55 Wang Y, Abarbanell AM, Herrmann JL, *et al.* TLR4 inhibits mesenchymal stem cell (MSC) STAT3 activation and thereby exerts deleterious effects on MSC-mediated cardioprotection. *PLoS ONE* 2010;**5**(12):e14206.

56 Yagi H, Soto-Gutierrez A, Navarro-Alvarez N, *et al.* Reactive bone marrow stromal cells attenuate systemic inflammation via sTNFR1. *Mol Ther* 2010;**18**(10):1857–1864.

57 Puglisi MA, Tesori V, Lattanzi W, *et al.* Therapeutic implications of mesenchymal stem cells in liver injury. *Biomed Biotechnol* 2011;**2011**:860578.

58 Li Q, Yao D, Ma J. Transplantation of MSCs in combination with netrin-1 improves neoangiogenesis in a rat model of hind limb ischemia. *J Surg Res* 2011;**166**(1):162–169.

59 Adamowicz J, Pokrywczyńska M, Drewa T. Conditioned medium derived from mesenchymal stem cells culture as a intravesical therapy for cystitis interstitials. *Med Hypotheses* 2014;**2**(6):670–673.

60 Yagi H, Soto-Gutierrez A, Kitagawa Y, *et al.* Bone marrow mesenchymal stromal cells attenuate organ injury induced by LPS and burn. *Cell Transplant* 2010;**19**(6):823–830.

61 Salomone F, Barbagallo I, Puzzo L, *et al.* Efficacy of adipose tissue-mesenchymal stem cell transplantation in rats with acetaminophen liver injury. *Stem Cell Res* 2013;**11**(3):1037–1044.

62 Kanazawa H, Fujimoto Y, Teratani T, *et al.* Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. *PLoS ONE* 2011;**6**(4):e19195.

63 Kim HS, Choi DY, Yun SJ, *et al.* Proteomic analysis of microvesicles derived from human mesenchymal stem cells. *J Proteome Res* 2012;**11**(2):839–849.

64 Bartholomew A, Sturgeon C, Siatskas M, *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;**30**:42–48.

65 Le Blanc K, Rasmusson I, Sundberg B, *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–1441.

66 Lee JW, Fang X, Gupta N, *et al.* Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the *ex vivo* perfused human lung. *Proc Natl Acad Sci U S A* 2009;**106**:16357–16362.

67 Elman JS, Li M, Wang F, *et al.* A comparison of adipose and bone marrow-derived mesenchymal stromal cell secreted factors in the treatment of systemic inflammation. *J Inflamm (Lond)* 2014;**11**(1):1.

68 Eliopoulos N, Zhao J, Bouchentouf M, *et al.* Human marrow-derived mesenchymal stromal cells decrease cisplatin renotoxicity *in vitro* and *in vivo* and enhance survival of mice post-intraperitoneal injection. *Am J Physiol Renal Physiol* 2010;**299**(6):F1288–F1298.

69 Danchuk S, Ylostalo JH, Hossain F, *et al.* Human multipotent stromal cells attenuate lipopolysaccharide-induced acute lung injury in mice via secretion of tumor necrosis factor-α-induced protein 6. *Stem Cell Res Ther* 2011;**2**(3):27.

70 Yarilin AA. *Osnovy Immunologii* [*Basics of Immunology*]. Meditsina: Moscow, 1999.

71 Groh ME, Maitra B, Szekely E, Koç ON. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol* 2005;**33**:928–934.

72 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.

73 Sotiropoulou PA, Perez SA, Gritzapis AD, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**(1):74–85.

74 Montzka K, Führmann T, Müller-Ehmsen J, *et al.* Growth factor and cytokine expression of human mesenchymal stromal cells is not altered in an *in vitro* model of tissue damage. *Cytotherapy* 2010;**12**:870–880.

75 Hwang SH, Cho HK, Park SH, *et al.* Toll like receptor 3 & 4 responses of human turbinate derived mesenchymal stem cells: stimulation by double stranded RNA and lipopolysaccharide. *PLoS ONE* 2014;**9**(7):e101558.

76 Waterman RS, Tomchuck SL, Henkle SL, Betancourt AM. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* 2010;**5**(4):e10088.

77 Chen Z, He X, He X, *et al.* Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun* 2014;**450**(4):1402–1408.

78 Kehoe O, Cartwright A, Askari A, *et al.* Intra-articular injection of mesenchymal stem cells leads to reduced inflammation and cartilage damage in murine antigen-induced arthritis. *J Transl Med* 2014;**12**:157.

79 Wu HJ, Yiu WH, Li RX, *et al.* Mesenchymal stem cells modulate albumin-induced renal tubular inflammation and fibrosis. *PLoS ONE* 2014;**9**(3):e90883.

80 Blundell CD, Mahoney DJ, Almond A, *et al.* The link module from ovulation- and inflammation-associated protein TSG-6 changes conformation on hyaluronan binding. *J Biol Chem* 2003;**278**(49):49261–49270.

81 Lee RH, Pulin AA, Seo MJ, *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**(1):54–63.

82 Wang N, Li Q, Zhang L, *et al.* Mesenchymal stem cells attenuate peritoneal injury through secretion of TSG-6. *PLoS ONE* 2012;**7**(8):e43768.

83 Roddy GW, Oh JY, Lee RH, *et al.* Action at a distance: systematically administered adult stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment and primarily by secretion of TNF-α stimulated gene/protein 6. *Stem Cells* 2011;**29**(10):1572–1579.

84 Bonfield TL, Nolan (Koloze) MT, Lennon DP, Caplan AI. *Defining human mesenchymal stem cell efficacy in vivo. J Inflamm (Lond)* 2010;**7**:51.

85 Tomchuck SL, Zwezdaryk KJ, Coffelt SB, *et al.* Toll-like receptors on human mesenchymal stem cells drive their migration and immunomodulating responses. *Stem Cells* 2008;**26**(1):99–107.

86 Verreck FA, de Boer T, Langenberg DM, *et al.* Phenotypic and functional profiling of human proinflammatory type-1 and anti-inflammatory type-2 macrophages in response to microbial antigens and IFN-γ- and CD40L-mediated costimulation. *J Leukoc Biol* 2006;**79**(2):285–293.

87 Qiu Y, Yun MM, Han X, *et al.* Human umbilical cord mesenchymal stromal cells suppress MHC class II expression on rat vascular endothelium and prolong survival time of cardiac allograft. *Int J Clin Exp Med* 2014;**7**(7):1760–1767.

88 Chen YT, Sun CK, Lin YC, *et al.* Adipose-derived mesenchymal stem cell protects kidneys against ischemia–reperfusion injury through suppressing oxidative stress and inflammatory reaction. *J Transl Med* 2011;**9**:51.

89 Peng Y, Chen X, Liu Q, *et al.* Mesenchymal stromal cells infusions improve refractory chronic graft versus host disease through an increase of $CD5^+$ regulatory B cells producing interleukin 10. *Leukemia* 2015;**29**(3):636–646.

90 Kwon MS, Noh MY, Oh KW, *et al.* The immunomodulatory effects of human mesenchymal stem cells on peripheral blood mononuclear cells in ALS patients. *J Neurochem* 2014;**131**:206–218.

91 Oh JY, Kim MK, Shin MS, *et al.* Cytokine secretion by human mesenchymal stem cells cocultured with damaged corneal epithelial cells. *Cytokine* 2009;**46**(1):100–103.

92 Parekkadan B, van Poll D, Megeed Z, *et al.* Immunomodulation of activated hepatic stellate cells by mesenchymal stem cells. *Biochem Biophys Res Commun* 2007;**363**(2):247–252.

93 Roemeling-van Rhijn M, Khairoun M, Korevaar SS, *et al.* Human bone marrow- and adipose tissue-derived mesenchymal stromal cells are immunosuppressive *in vitro* and in a humanized allograft rejection model. *J Stem Cell Res Ther* 2013;**S6**(1):20780.

94 Leijs MJ, van Buul GM, Lubberts E, *et al.* Effect of arthritic synovial fluids on the expression of immunomodulatory factors by mesenchymal stem cells: an explorative *in vitro* study. *Front Immunol* 2012;**3**:231.

95 Wen L, Zhu M, Madigan MC, *et al.* Immunomodulatory effects of bone marrow-derived mesenchymal stem cells on pro-inflammatory cytokine-stimulated human corneal epithelial cells. *PLoS ONE* 2014;**9**(7):e101841.

96 Levin S, Pevsner-Fischer M, Kagan S, *et al.* Divergent levels of LBP and TGFβ1 in murine MSCs lead to heterogenic response to TLR and proinflammatory cytokine activation. *Stem Cell Rev* 2014;**10**(3):376–388.

97 Goolaerts A, Pellan-Randrianarison N, Larghero J, *et al.* Conditioned media from mesenchymal stromal cells restore sodium transport and preserve epithelial permeability in an *in vitro* model of acute alveolar injury. *Am J Physiol Lung Cell Mol Physiol* 2014;**306**(11):L975–L985.

98 Bhargava R, Janssen W, Altmann C, *et al.* Intratracheal IL-6 protects against lung inflammation in direct, but not indirect, causes of acute lung injury in mice. *PLoS ONE* 2013;**8**(5):e61405.

99 Crisostomo PR, Wang M, Herring CM, *et al.* Gender differences in injury induced mesenchymal stem cell apoptosis and VEGF, TNF, IL-6 expression: role of the 55 kDa TNF receptor (TNFR1). *J Mol Cell Cardiol* 2007;**42**(1):142–149.

100 Leskela HV, Olkku A, Lehtonen S, *et al.* Estrogen receptor alpha genotype confers interindividual variability of response to estrogen and testosterone in mesenchymal-stem-cell-derived osteoblasts. *Bone* 2006;**39**(5):1026–1034.

101 Wang Q, Yu JH, Zhai HH, *et al.* Temporal expression of estrogen receptor alpha in rat bone marrow mesenchymal stem cells. *Biochem Biophys Res Commun* 2006;**347**(1):117–123.

102 Han HJ, Heo JS, Lee YJ. Estradiol-17β stimulates proliferation of mouse embryonic stromal cells: involvement of MAPKs and CDKs as well as protooncogenes. *Am J Physiol Cell Physiol* 2006;**290**(4): C1067–C1075.

103 Di Nicola M, Carlo-Stella C, Magni M, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.

104 Batten P, Sarathchandra P, Antoniw JW, *et al.* Human mesenchymal stem cells induce T cell anergy and downregulate T cell allo-responses via the TH2 pathway: relevance to tissue engineering human heart valves. *Tissue Eng* 2006;**12**:2263–2273.

105 Meisel R, Zibert A, Laryea M, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**:4619–4621.

106 Corcione A, Benvenuto F, Ferretti E, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**:367–372.

107 Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007;**25**(10):2648–2659.

108 Kinnaird T, Stabile E, Burnett MS, *et al.* Local delivery of marrow-derived stromal cells augments collateral perfusion through paracrine mechanisms. *Circulation* 2004;**109**:1543–1549.

109 Zhu Y, Yang Y, Zhang Y, *et al.* Placental mesenchymal stem cells of fetal and maternal origins demonstrate different therapeutic potentials. *Stem Cell Res Ther* 2014;**5**(2):48.

110 Takehara Y, Yabuuchi A, Ezoe K, *et al.* The restorative effects of adipose-derived mesenchymal stromal cells on damaged ovarian function. *Lab Invest* 2013;**93**(2):181–193.

111 Oh JY, Kim MK, Shin MS, *et al.* The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* 2008;**26**(4):1047–1055.

112 Kumar P, Miller AI, Polverini PJ. p38 MAPK mediates γ-irradiation-induced endothelial cell apoptosis, and vascular endothelial growth factor protects endothelial cells through the phosphoinositide 3-kinase–Akt–Bcl-2 pathway. *J Biol Chem* 2004;**279**:43352–43360.

113 Alberts B, Johnson A, Lewis J, *et al. Molecular Biology of the Cell.* Garland Science: New York, 2002.

114 Lieberthal W, Triaca V, Levine J. Mechanisms of death induced by cisplatin in proximal tubular epithelial cells: apoptosis vs. necrosis. *Am J Physiol* 1996;**270**:F700–F708.

115 García-Fernández M, Delgado G, Puche JE, *et al.* Low doses of insulin-like growth factor I improve insulin resistance, lipid metabolism, and oxidative damage in aging rats. *Endocrinology* 2008;**149**:2433–2442.

116 Okada M, Sugita K, Inukai T, *et al.* Hepatocyte growth factor protects small airway epithelial cells from apoptosis induced by tumor necrosis factor-α or oxidative stress. *Pediatr Res* 2004;**56**:336–344.

117 Yin F, Guo L, Meng CY, *et al.* Transplantation of mesenchymal stem cells exerts anti-apoptotic effects in adult rats after spinal cord ischemia–reperfusion injury. *Brain Res* 2014;**1561**:1–10.

118 Sarhan M, El Serougy H, Hussein AM, *et al.* Impact of bone-marrow-derived mesenchymal stem cells on Adriamycin-induced chronic nephropathy. *Can J Physiol Pharmacol* 2014;**92**(9): 733–743.

119 Chen M, Xiang Z, Cai J. The anti-apoptotic and neuro-protective effects of human umbilical cord blood mesenchymal stem cells (hUCB-MSCs) on acute optic nerve injury is transient. *Brain Res* 2013;**1532**:63–75.

120 Baglio SR, Pegtel DM, Baldini N. Mesenchymal stem cell secreted vesicles provide novel opportunities in (stem) cell-free therapy. *Front Physiol* 2012;**3**:359.

121 Bruno S, Grange C, Collino F, *et al.* Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PLoS ONE* 2012;**7**(3):e33115.

122 Bruno S, Grange C, Deregibus MC, *et al.* Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;**20**:1053–1067.

123 Gatti S, Bruno S, Deregibus MC, *et al.* Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia–reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant* 2011;**26**:1474–1483.

124 Reis LA, Borges FT, Simões MJ, *et al.* Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats. *PLoS ONE* 2012;**7**(9):e44092.

125 Herrera MB, Fonsato V, Gatti S, *et al.* Human liver stem cell-derived microvesicles accelerate hepatic regeneration in hepatectomized rats. *J Cell Mol Med* 2010;**14**(6b):1605–1618.

126 Tan CY, Lai RC, Wong W, *et al.* Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Steml Cell Res Ther* 2014;**5**(3):76.

127 Lai RC, Arslan F, Lee MM, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4** (3):214–222.

128 Bian S, Zhang L, Duan L, *et al.* Extracellular vesicles derived from human bone marrow mesenchymal stem cells promote angiogenesis in a rat myocardial infarction model. *J Mol Med (Berl)* 2014;**92** (4):387–397.

129 Sdrimas K, Kourembanas S. MSC microvesicles for the treatment of lung disease: a new paradigm for cell-free therapy. *Antioxid Redox Signal* 2014;**21**(13):1905–1915.

130 Ratajczak J, Miekus K, Kucia M, *et al.* Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;**20**(5):847–856.

131 Chen TS, Lai RC, Lee MM, *et al.* Mesenchymal stem cell secretes microparticles enriched in pre-micro RNAs. *Nucleic Acids Res* 2010;**38**,215–224.

132 Collino F, Bruno S, Deregibus MC, *et al.* MicroRNAs and mesenchymal stem cells. *Vitam Horm* 2011;**87**:291–320.

133 Galitskiĭ VA. Hypothesis of the initiation of DNA methylation *de novo* and allelic exclusion by small RNA. *Tsitologiia* 2008;**50**(4):277–286.

134 Collino F, Deregibus MC, Bruno S, *et al.* Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stromal cells shuttle selected pattern of miRNAs. *PLoS ONE* 2010;**5**(7):e11803.

135 Zhu YG, Feng XM, Abbott J, *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;**32**(1):116–125.

136 Jaeschke H. Mechanisms of reperfusion injury after warm ischemia of the liver. *J Hepatobiliary Pancreat Surg* 1998;**5**(4):402–408.

137 Teoh NC, Farrell GC. Hepatic ischemia reperfusion injury: pathogenic mechanisms and basis for hepatoprotection. *J Gastroenterol Hepatol* 2003;**18**(8):891–902.

138 Jaeschke H. Molecular mechanisms of hepatic ischemia–reperfusion injury and preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2003;**284**(1):G15–G26.

139 Girn HR, Ahilathirunayagam S, Mavor AI, Homer-Vanniasinkam S. Reperfusion syndrome: cellular mechanisms of microvascular dysfunction and potential therapeutic strategies. *Vasc Endovascular Surg* 2007;**41**(4):277–293.

140 Wu J, Li J, Zhang N, Zhang C. Stem cell-based therapies in ischemic heart diseases: a focus on aspects of microcirculation and inflammation. *Basic Res Cardiol* 2011;**106**(3):317–324.

141 Arslan F, de Kleijn DP, Timmers L, *et al.* Bridging innate immunity and myocardial ischemia/reperfusion injury: the search for therapeutic targets. *Curr Pharm Des* 2008;**14**:1205–1216.

142 Engler RL, Dahlgren MD, Peterson MA, *et al.* Accumulation of polymorphonuclear leukocytes during 3-h experimental myocardial ischemia. *Am J Physiol* 1986;**251**:H93–H100.

143 Gao X, Zhang H, Belmadani S, *et al.* Role of TNF-α-induced reactive oxygen species in endothelial dysfunction during reperfusion injury. *Am J Physiol Heart Circ Physiol* 2008;**295**:H2242–H2249.

144 Zhang C, Wu J, Xu X, *et al.* Direct relationship between levels of TNF-α expression and endothelial dysfunction in reperfusion injury. *Basic Res Cardiol* 2010;**105**:453–464.

145 Chang EL, Lee SH, Mun KC, *et al.* Effect of artificial cells on hepatic function after ischemia–reperfusion injury in liver. *Transplant Proc* 2004;**36**(7):1959–1961.

146 Sologub TV, Romanzov MG, Kremen NV. *Free-Radical Processes and Inflammation (Pathogenic, Clinical and Therapeutic Aspects). Textbook for Medical Doctors*. Academiya Estestvoznania: Moscow, 2008.

147 Liu H, Liu S, Li Y, *et al.* The role of SDF-1–CXCR4/CXCR7 axis in the therapeutic effects of hypoxia-preconditioned mesenchymal stromal cells for renal ischemia/reperfusion injury. *PLoS ONE* 2012;**7**(4):e34608.

148 Khubutia MSh, Temnov AA, Vagabov AV, *et al.* Stromal cell derived low-molecular peptide in treatment of acute renal failure. *Transplantologia* 2011;**4**:20–25.

149 Sachse A, Wolf G. Angiotensin II-induced reactive oxygen species and the kidney. *J Am Soc Nephrol* 2007;**18**:2439–2446.

150 Feng Z, Ting J, Alfonso Z, *et al.* Fresh and cryopreserved, uncultured adipose tissue-derived stem and regenerative cells ameliorate ischemia–reperfusion-induced acute kidney injury. *Nephrol Dial Transplant* 2010;**25**:3874–3884.

151 Tomita, S, Mickle, DA, Weisel RD, *et al.* Improved heart function with myogenesis and angiogenesis after autologous porcine bone marrow stromal cell transplantation. *J Thorac Cardiovasc Surg* 2002;**123**:1132–1140.

152 Davani S, Marandin A, Mersin N, *et al.* Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* 2003;**108**(Suppl 1):II253–II258.

153 Wang JS, Shum-Tim D, Chedrawy E, Chiu RC. The coronary delivery of marrow stromal cells for myocardial regeneration: pathophysiologic and therapeutic implications. *J Thorac Cardiovasc Surg* 2001;**122**:699–705.

154 Armiñán A, Gandia C, García-Verdugo JM, *et al.* Mesenchymal stromal cells provide better results than hematopoietic precursors for the treatment of myocardial infarction. *J Am Coll Cardiol* 2010;**55**:2244–2253.

155 Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004;**95**:9–20.

156 Gnecchi M, He H, Noiseux N. Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* 2006;**20**:661–669.

157 Haider HKh, Ashraf M. Bone marrow stem cell transplantation for cardiac repair. *Am J Physiol Heart Circ Physiol* 2005;**288**:H2557–H2567.

158 Kudo M, Wang Y, Wani MA, *et al.* Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. *J Mol Cell Cardiol* 2003;**35**:1113–1119.

159 Gnecchi M, He H, Liang OD, *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;**11**:367–368.

160 Togel F, Hu Z, Weiss K, *et al.* Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 2005;**289**:F31–F42.

161 Boyle AJ, McNiece IK, Hare JM. Mesenchymal stem cell therapy for cardiac repair. *Methods Mol Biol* 2010;**660**:65–84.

162 Angoulvant D, Ivanes F, Ferrera R, *et al.* Mesenchymal stem cell conditioned media attenuates *in vitro* and *ex vivo* myocardial reperfusion injury. *J Heart Lung Transplant* 2011;**30**(1):95–102.

163 Pevsner-Fischer M, Morad V, Cohen-Sfady M, *et al.* Toll-like receptors and their ligands control mesenchymal stromal cell functions. *Blood* 2007;**109**:1422–1432.

164 Rolls A, Shechter R, London A, *et al.* Toll-like receptors modulate adult hippocampal neurogenesis. *Nat Cell Biol* 2007;**9**:1081–1088.

165 Webber MJ, Han X, Murthy SN, *et al.* Capturing the stem cell paracrine effect using heparin-presenting nanofibers to treat cardiovascular diseases. *J Tissue Eng Regen Med* 2010;**4**(8):600–610.

166 Sun CK, Yen CH, Lin YC, *et al.* Autologous transplantation of adipose-derived mesenchymal stem cells markedly reduced acute ischemia–reperfusion lung injury in a rodent model. *J Transl Med* 2011;**9**:118.

167 Chen S, Chen L, Wu X. Ischemia postconditioning and mesenchymal stem cells engraftment synergistically attenuate ischemia reperfusion-induced lung injury in rats. *J Surg Res* 2012;**178**(1):81–91.

168 Manning E, Pham S, Li S, *et al.* Interleukin-10 delivery via mesenchymal stem cells: a novel gene therapy approach to prevent lung ischemia-reperfusion injury. *Hum Gene Ther* 2010;**21**(6):713–727.

169 Jiang H, Qu L, Li Y, *et al.* Bone marrow mesenchymal stem cells reduce intestinal ischemia/reperfusion injuries in rats. *J Surg Res* 2009;**168**(1):127–134.

170 Park SS, Caballero S, Bauer G, *et al.* Long-term effects of intravitreal injection of GMP-grade bone-marrow-derived $CD34^+$ cells in NOD-SCID mice with acute ischemia–reperfusion injury. *Invest Ophthalmol Vis Sci* 2012;**53**(2):986–994.

171 Dharmasaroja P. Bone marrow-derived mesenchymal stem cells for the treatment of ischemic stroke. *J Clin Neurosci* 2009;**16**:12–20.

172 Evans MJ, Van Winkle LS, Fanucchi MV, Plopper CG. Cellular and molecular characteristics of basal cells in airway epithelium. *Exp Lung Res* 2001;**27**(5):401–415.

173 Zhu Y, Ehre C, Abdullah LH, *et al.* Munc13-$2^{-/-}$ baseline secretion defect reveals source of oligomeric mucins in mouse airways. *J Physiol* 2008;**586**(7):1977–1992.

174 Rock JR, Onaitis MW, Rawlins EL, *et al.* Basal cells as stem cells of the mouse trachea and human airway epithelium. *Proc Natl Acad Sci U S A*. 2009;**106**(31):12771–12775.

175 Blatt EN, Yan XH, Wuerffel MK, *et al.* Forkhead transcription factor HFH-4 expression is temporally related to ciliogenesis. *Am J Respir Cell Mol Biol* 1999;**21**(2):168–176.

176 You Y, Huang T, Richer EJ, *et al.* Role of f-box factor foxj1 in differentiation of ciliated airway epithelial cells. *Am J Physiol Lung Cell Mol Physiol* 2004;**286**(4):L650–L657.

177 Volckaert T, De Langhe S. Lung epithelial stromal cells and their niches: Fgf10 takes center stage. *Fibrogenesis Tissue Repair* 2014;**7**:8.

178 Hegab AE, Nickerson DW, Ha VL. Repair and regeneration of tracheal surface epithelium and submucosal glands in a mouse model of hypoxic-ischemic injury. *Respirology* 2012;**17**(7):1101–1113.

179 Hegab AE, Ha VL, Gilbert JL, *et al.* Novel stem/progenitor cell population from murine tracheal submucosal gland ducts with multipotent regenerative potential. *Stem Cells* 2011;**29**(8):1283–1293.

180 Reynolds SD, Hong KU, Giangreco A, *et al.* Conditional Clara cell ablation reveals a self-renewing progenitor function of pulmonary neuroendocrine cells. *Am J Physiol Lung Cell Mol Physiol* 2000;**278**(6):L1256–L1263.

181 Hsu HS, Liu CC, Lin JH, *et al.* Repair of naphthalene-induced acute tracheal injury by basal cells depends on β-catenin. *J Thorac Cardiovasc Surg* 2014;**148**(1):322–332.

182 Giangreco A, Reynolds SD, Stripp BR. Terminal bronchioles harbor a unique airway stem cell population that localizes to the bronchoalveolar duct junction. *Am J Pathol* 2002;**161**(1):173–182.

183 Reynolds SD, Giangreco A, Power JH, Stripp BR. Neuroepithelial bodies of pulmonary airways serve as a reservoir of progenitor cells capable of epithelial regeneration. *Am J Pathol* 2000;**156**(1):269–278.

184 Gomperts BN, Belperio JA, Rao PN, *et al.* Circulating progenitor epithelial cells traffic via CXCR4/CXCL12 in response to airway injury. *J Immunol* 2006;**176**(3):1916–1927.

185 Gomperts BN, Belperio JA, Fishbein MC, *et al.* Keratinocyte growth factor improves repair in the injured tracheal epithelium. *Am J Respir Cell Mol Biol* 2007;**37**(1):48–56.

186 Theise ND, Henegariu O, Grove J, *et al.* Radiation pneumonitis in mice: a severe injury model for pneumocyte engraftment from bone marrow. *Exp Hematol* 2002;**30**(11):1333–1338.

187 Gupta N, Su X, Popov B, *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;**179**(3):1855–1863.

188 Ortiz LA, Gambelli F, McBride C, *et al.* Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**(14):8407–8411.

189 Rojas M, Xu J, Woods CR, *et al.* Bone marrow derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* 2005;**33**(2):145–52.

190 Tadokoro T, Wang Y, Barak LS, *et al.* IL-6/STAT3 promotes regeneration of airway ciliated cells from basal stem cells. *Proc Natl Acad Sci U S A* 2014;**111**(35):E3641–E3649.

191 Steinemann D, Göhring G, Schlegelberger B. Genetic instability of modified stem cells –a first step towards malignant transformation? *Am J Stem Cells* 2013;**2**(1):39–51.

192 Izadpanah R, Kaushal D, Kriedt C, *et al.* Long-term *in vitro* expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 2008;**68**(11):4229–4238.

193 Sundin M, Orvell C, Rasmusson I, *et al.* Mesenchymal stem cells are susceptible to human herpesviruses, but viral DNA cannot be detected in the healthy seropositive individual. *Bone Marrow Transplant* 2006;**37**(11):1051–1059.

194 Amariglio N, Hirshberg A, Scheithauer BW, *et al.* Donor-derived brain tumor following neural stem cell transplantation in an ataxia telangiectasia patient. *PLoS Med* 2009;**6**(2):e1000029.

195 Lund RD, Kwan AS, Keegan DJ, *et al.* Cell transplantation as a treatment for retinal disease. *Prog Retin Eye Res* 2001;**20**(4):415–449.

196 Lund RD, Ono SJ, Keegan DJ, Lawrence JM. Retinal transplantation: progress and problems in clinical application. *J Leukoc Biol* 2003;**74**(2):151–160.

197 Klassen H, Sakaguchi DS, Young MJ. Stem cells and retinal repair. *Prog Retin Eye Res* 2004;**23**(2):149–181.

198 Aramant RB, Seiler MJ. Progress in retinal sheet transplantation. *Prog Retin Eye Res* 2004;**23**(5):475–494.

199 Das AM, Zhao X, Ahmad I. Stem cell therapy for retinal degeneration: retinal neurons from heterologous sources. *Semin Ophthalmol* 2005;**20**(1):3–10.

200 Ikeda H, Osakada F, Watanabe K, *et al.* Generation of $Rx^+/Pax6^+$ neural retinal precursors from embryonic stem cells. *Proc Natl Acad Sci U S A* 2005;**102**(32):11331–11336.

201 Banin E, Obolensky A, Idelson M, *et al.* Retinal incorporation and differentiation of neural precursors derived from human embryonic stem cells. *Stem Cells* 2006;**24**(2):246–257.

202 Böhm MR, Pfrommer S, Chiwitt C, *et al.* Crystallin-β-b2-overexpressing NPCs support the survival of injured retinal ganglion cells and photoreceptors in rats. *Invest Ophthalmol Vis Sci* 2012;**53**(13):8265–8279.

203 Cramer AO, McLaren RE. Translating induced pluripotent stem cells from bench to bedside: application to retinal deceases. *Curr Gene Ther* 2013;**13**(2):139–151.

204 McConnell MP, Dhar S, Naran S, *et al. In vivo* induction and delivery of nerve growth factor, using HEK-293 cells. *Tissue Eng* 2004;**10**(9–10):1492–1501.

205 Jimenez JC, Tyson DR, Dhar S, *et al.* Human embryonic kidney cells (HEK-293 cells): characterization and dose–response relationship

for modulated release of nerve growth factor for nerve regeneration. *Plast Reconstr Surg* 2004;**113**(2):605–610.

206 Odorico JS, Kaufman DS, Thomson JA. Multilineage differentiation from human embryonic stromal cell lines. *Stem Cells* 2001;**19**(3):193–204.

207 Kawasaki H, Suemori H, Mizuseku K, *et al.* Generation of dopaminergic neurons and pigmented epithelia from primate ES cells by stromal cell-derived inducing activity. *Proc Natl Acad Sci U S A* 2002;**99**(3):1580–1585.

208 Germain ND, Hartman NW, Cai C, *et al.* Teratocarcinoma formation in embryonic stem cell-derived neural progenitor hippocampal transplants. *Cell Transplant* 2012;**21**(8):1603–1611.

209 Gordeeva OF, Nikonova TM. Development of experimental tumors formed by mouse and human embryonic stem and teratocarcinoma cells after subcutaneous and intraperitoneal transplantations into immunodeficient and immunocompetent mice. *Cell Transplant* 2013;**22**(10):1901–1914.

210 Uteza Y, Rouillot JS, Kobetz A, *et al.* Intravitreous transplantation of encapsulated fibroblasts secreting the human fibroblast growth factor 2 delays photoreceptor cell degeneration in Royal College of Surgeons rats. *Proc Natl Acad Sci U S A* 1999;**96**(6):3126–3131.

211 Bush RA, Lei B, Tao W, *et al.* Encapsulated cell-based intraocular delivery of ciliary neurotrophic factor in normal rabbit: dose-dependent effects on ERG and retinal histology. *Invest Ophthalmol Vis Sci* 2004;**45**(7):2420–2430.

212 Sieving PA, Caruso RC, Tao W, *et al.* Ciliary neurotrophic factor (CNTF) for human retinal degeneration: phase I trial of CNTF delivered by encapsulated cell intraocular implants. *Proc Natl Acad Sci U S A* 2006;**103**(10):3896–3901.

213 Arbab AS, Jordan EK, Wilson LB, *et al. In vivo* trafficking and targeted delivery of magnetically labeled stromal cells. *Hum Gene Ther* 2004;**15**(4):351–360.

214 Song M, Kim YJ, Kim YH, *et al.* Using a neodymium magnet to target delivery of ferumoxide-labeled human neural stem cells in a rat model of focal cerebral ischemia. *Hum Gene Ther* 2010;**21**(5):603–610.

215 Kyrtatos PG, Lehtolainen P, Junemann-Ramirez M, *et al.* Magnetic tagging increases delivery of circulating progenitors in vascular injury. *JACC Cardiovasc Interv* 2009;**2**(8):794–802.

216 Yanai A, Häfeli UO, Metcalfe AL, *et al.* Focused magnetic stem cell targeting to the retina using superparamagnetic iron oxide nanoparticles. *Cell Transplant* 2012;**21**(6):1137–1148.

CHAPTER 36

# The use of mesenchymal stromal cells in the treatment of diseases of the cornea

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## 36.1 Introduction

As a key component of the ocular surface required for vision, the cornea has been extensively studied as a site for cell and tissue-based therapies. Historically, these treatments have consisted of donor corneal tissue transplants [1], but cultivated epithelial autografts have become established over the last 15 years as a routine treatment for ocular surface disease [2–7]. Ultimately, these treatments are performed with the intention of restoring corneal transparency and a smooth ocular surface. The degree of success, however, is often dependent upon the inherent level of corneal inflammation at time of treatment [6,7]. In this regard, the anti-inflammatory and immunomodulatory properties of mesenchymal stromal cells (MSCs) have drawn attention to these cells as potential therapeutic agents for corneal repair.

The origins for MSC-based therapies are founded in part on observations of the recruitment of endogenous bone-marrow-derived cells to injured corneas [8,9]. However, an increasing quantity of data is emerging for MSC administration following their isolation and *ex vivo* expansion from a variety of tissues, including bone marrow [10,11], adipose tissue [12], umbilical cord [13], and dental pulp [14]. In brief, evidence has emerged of cultured MSCs, or their secreted products, having a positive impact on corneal wound healing (Table 36.1) and on retention of corneal allografts (Table 36.2) in animal models.

Optimal dosage, route and timing of administration, however, all remain active areas of investigation. Intriguingly, amidst these studies have emerged reports of MSC transdifferentiation into corneal cells (Table 36.3). The clearest evidence has been obtained with respect to expression of markers associated with the phenotype of corneal stromal cells. In contrast, the evidence for MSC conversion to corneal epithelial cell types remains inconclusive. In neither case does the conversion of

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**Table 36.1** Effect of MSCs on corneal wound healing.

| Reference | MSC source | Species | Treatment | Observations |
|---|---|---|---|---|
| Ye *et al.* [11] | Bone marrow | Rabbit | DiI-labeled cells were applied intravenously following alkali burn to cornea. | Labeled cells trafficked to all cell layers of the cornea within 14 days. Enhanced expression of epithelial regeneration markers observed in limbus and peripheral cornea. |
| Oh *et al.* [69] | Bone marrow | Rat | Applied topically to alcohol-denuded corneas. | Dose-dependent reduction in inflammation and neovascularization. Modulations in immune infiltrates were consistent with physiological remodeling and regeneration. Elevations in IL-10, TGF-β1, and TSP-1, coupled with decreases in IL-2, IFN-γ, and CD4, were indicative of reduced inflammatory load. |
| Monteiro *et al.* [14] | Dental pulp | Human | Cultured sheets applied to alkali-wounded rabbit corneas and covered with amniotic membrane. | MSC-treated eyes showed marked improvement compared with control eyes treated with amniotic membrane alone. |
| Liu *et al*. [13] | Umbilical cord | Human | Injected into thin and cloudy corneas of lumican null mutant and keratocan null mutant mice. | Increased corneal thickness and clarity. |
| Zajicova *et al.* [70] | Bone marrow | Mouse | Applied to surgically denuded ocular surface of mice in conjunction with polyamide scaffold and limbal epithelial cells. | Reduction in inflammatory cytokines (IFN-γ, IL-2, iNOS) observed by RT-qPCR. |
| Gomes *et al.* [71] | Dental pulp | Human | Cultured sheets applied to alkali-wounded rabbit corneas and covered with amniotic membrane. | Reduced neovascularization and improved re-epithelialization. |
| Roddy *et al.* [72] | Bone marrow | Human | Applied intravenously or via intraperitoneal injection into rats with alcohol-denuded corneal epithelium. | Reduction in corneal opacity and neutrophil recruitment. Reduction in production of proinflammatory cytokines (including IL-1β) and the angiogenic factor Tenascin C. Role identified for TSG-6. |
| Agorogianni *et al.* [19] | Adipose tissue (autologous) | Human | Applied topically to nonhealing ulcer. | Healing of ulcer observed. |
| Yao *et al.* [73] | Bone marrow | Rat | Subconjunctival injection into rats with alkali-wounded corneas. | Improved re-epithelialization. Reduced infiltration by $CD68^+$ macrophage. Reduced expression of MIP-1α and TNF-α. |
| Cejkova *et al.* [74] | Bone marrow | Rabbit | Applied to ocular surface of alkali-wounded corneas in conjunction with a nanoporous PLA scaffold. | Decreased expression of proinflammatory cytokines, including IL-1β, IL-2, and IFN-γ. Reduced corneal neovascularization accompanied by a reduction in VEGF expression. |
| Lin *et al.* [75] | Adipose tissue | Human | Subconjunctival injection into rabbits with alkali-wounded corneas. | Improved re-epithelialization. |
| Zeppieri *et al.* [76] | Adipose tissue | Human | Applied to ocular surface of alkali-wounded rat corneas. | Reduction in inflammatory cells and improved re-epithelialization. |
| Pinarli *et al.* [77] | Bone marrow | Rat | Grown on amniotic membrane and applied to denuded corneal surface. | Improved re-epithelialization. |
| Jarvorkova *et al.* [78] | Bone marrow | Rat | Systemic administration via an intravenous catheter | Evidence of homing to the site of ocular injury. MSCs pretreated with IFN-γ reduced levels of IL-1α, IL-6, and iNOS in the damaged eye. |
| Lee *et al.* [79] | Bone marrow | Mouse | Systemic administration via intraperitoneal injection | Reduction in $CD4^+$ and $CD8^+$ lymphocytes of the draining lymph nodes. |

CD4: cluster of differentiation 4; CD8: cluster of differentiation 8; CD68: cluster of differentiation 68; DiI: a fluorescent lipophilic dialkylcarbocyanine dye for labeling cells; iNOS: inducible nitric oxide synthase; MIP-1α: macrophage inflammatory protein 1 α; PLA: poly(lactic acid); RT-qPCR: reverse transcriptase–quantitative (real-time) polymerase chain reaction; TNF-α: tumor necrosis factor α; TSG-6: tumor necrosis factor α-stimulating gene/protein 6; TSP-1: thrombospondin 1; VEGF: vascular endothelial growth factor.

**Table 36.2** Effect of MSCs on corneal allograft survival, cytokine production, and immune cell activation.

| Reference | MSC source and treatment | Transplantation model | Effect on transplant | Effect on lymphokine profiles | Other comments |
|---|---|---|---|---|---|
| Oh *et al.* [80] | Allogeneic rat MSCs applied topically. | Xenograft. Pig into rat. | No effect | No effect on IL-2 and IFN-γ. Increase in IL-6 and IL-10. | PKH-26-labeled MSCs were identified in the cornea, but failed to suppress infiltration by immune cells. |
| Jia *et al.* [81] | Allogeneic rat MSCs applied intravenously with and without CSP. | Allograft. Wistar into Lewis rats. | Increased survival when MSC applied alone or with ≥2 mg/kg CSP A. | Reduction: IL-2 and IFN-γ. Increase: IL-4 and positive trend for IL-10. | MSC therapy preoperation or postrejection had no effect. Effect of MSC therapy altered by CSP dose: 1 mg/kg, accelerated rejection; 2 mg/kg, decreased rejection. |
| Oh *et al.* [82] | Xenogeneic. Human MSC applied intravenously. | Allograft. B6 into BALB/c mice. | Increased graft survival. | Reduction: IL-6, IL-1β, IL-12a, IL-2, and IFN-γ. | Majority of cells lodge in lungs. Marked reduction in inflammatory cells within cornea, including $CD3^+$ T cells. Reduced activation of APC in cornea and DLN. Results linked to expression of TSG-6. |
| Lee *et al.* [83] | Xenogeneic. Human bone marrow MSCs applied intravenously. | Allograft. B6 into BALB/c mice. | Increased survival. | Reduction of IL-12a and IL-12b expression in DLN. | Reduced numbers of inflammatory cells within the cornea. Reduced numbers of $CCR7^+$ cells in lymph nodes. Reduction in chemokines involved with trafficking of APC to lymph nodes. |
| Omoto *et al.* [84] | Syngeneic. GFP-B6 into B6 or BALBc into BALB/c, applied intravenously. | Allograft. BALB/c into B6 or B6 into BALB/c. | Increased survival. | Syngeneic MSCs inhibited activation of IFN-γ-producing T cells. | GFP-B6 MSCs recruited to cornea, conjunctiva and DLNs. Reduction in $CD11c^+$/MHC $II^+$ DC maturation. |
| Treacy *et al.* [85] | Syngeneic or IFN-γ-treated syngeneic, or allogeneic or third-party. Lewis into Lewis, or Dark Agouti into Lewis, or Wistar-Furth into Lewis rats. All treatments applied intravenously via the tail vein. | Allograft. Dark Agouti grafts into Lewis rats. | Allogeneic and third-party MSCs prolong graft survival | Significant decreases in IL-6 and IL-1β in rats treated with allogeneic and third-part MSCs. | Reduction in the natural killer T cell numbers as well as APCs and $CD4^+CD25^+$ Tregs cells in MSC-treated animals. Importantly, the percentage of $Foxp3^+CD4^+CD25^+$ was increased in all MSC treatments except the IFN-γ-pre-treated cohort, where this T cell population was preferentially located in the spleen. Significantly increased levels of alloantibodies IgG1 and IgG2 following allogeneic MSC treatment were not related to graft survival. |

B6: C57 black 6 mouse strain; BALB/c: albino mouse strain; CCR7: C–C chemokine receptor 7; CD3: cluster of differentiation 3; CD11c: cluster of differentiation 11c; CD25: cluster of differentiation 25; CSP: cyclosporine; DLN: drainage lymph nodes, Foxp3: fork head box p3/scurfin; GFP: green fluorescent protein; PKH-26: a proprietary lipophilic dye used for labeling cell membranes.

**Table 36.3** Evidence for keratogenic differentiation of MSCs.

| Study | Species of MSC origin | Tissue of MSC origin | Study design | Host species | Observations |
|---|---|---|---|---|---|
| *Evidence for and against conversion into corneal epithelial cells* | | | | | |
| Guo *et al.* [87] | Human | Bone marrow | *In vivo* | Rabbit | Transplanted cells were retained on ocular surface and were immunoreactive toward antibodies to K12 and human antigen. |
| Gu *et al.* [88] | Rabbit | Bone marrow | *In vivo* | Rabbit | Evidence of survival by positive staining for BrdU-labeled cells. |
| | | | *In vitro* | | 10% of cells display immunostaining for K3 (mab AE5). |
| Ma *et al.* [86] | Human | Bone marrow | *In vivo* | Rat | Transplanted cells were retained on ocular surface but failed to express cytokeratin. |
| Monteiro e*t al.* [14] | Human | Dental pulp | *In vivo* | Rabbit | Transplanted cells were retained (human antigen) and co-expressed K3 (determined by IHC using mab AE5). |
| | | | *In vitro* | | Traces of K3/K12 detected in cultured cells by ICC (mab AE5), but only K12 detected by RT-PCR. |
| Gomes *et al.* [71] | Human | Dental pulp | *In vivo* | Rabbit | Transplanted cells were retained (human antigen) and expressed both K3 and K12 (IHC). |
| Zhang *et al.* [89] | Rat | Bone marrow | Organ culture | Dog | K3 was detected by RT-PCR and WB for cells grown on decellularized canine corneas, but levels were less than that for cultured limbal epithelial cell cultures. |
| Rohaina *et al.* [90] | Human | Bone marrow | *In vivo* | Rat | Fate of administered cells not tracked. |
| | | | *In vitro* | | Traces of K3 expression detected by ICC (mab AE5). |
| Katikireddy *et al.* [91] | Human | Bone marrow $SSEA4^{+/-}$ fractions compared | *In vitro* | — | K3 expression confirmed for $SSEA4^{+}$ fraction grown under epithelial growth conditions by ICC (mab AE5). Both K3 and K12 detected by RT-qPCR, but fivefold less than control tissue. |
| *Evidence for conversion into corneal stromal cells (keratocytes)* | | | | | |
| Du *et al.* [92] | Human | Adipose tissue (SP fraction) | *In vitro* | — | SP cells, grown under conditions to promote keratocyte differentiation, expressed keratocan. Expression confirmed by WB, RT-PCR, and RT-qPCR. |
| Liu *et al.* [13] | Human | Umbilical cord | *In vivo* | Mice $Lum^{-/-}$ $Kera^{-/-}$ | Labeled human cells ($DiI^{+}$) adopted keratocyte morphology and phenotype (keratocan, lumican, CD34) when implanted into corneal stromas of null mutant mice. Keratocan and lumican expression confirmed by WB. |
| Liu *et al.* [93] | Mouse | Bone marrow | *In vivo* | Mouse $Kera^{-/-}$ | Labeled cells ($DiI^{+}$) detected in stroma and co-localized with keratocan expression. |
| *Evidence for conversion into corneal endothelial cells* | | | | | |
| Liu and Zhao [94] | Rabbit | Bone marrow | *In vivo* | Rabbit Autologous | Evidence of reduced stromal edema, but phenotype of implanted cells not assessed. |
| Joyce *et al.* [95] | Human | Umbilical cord | Organ culture | Human | Labeled cells ($GFP^{+}$) became incorporated within wounded endothelium and a positive shift in gene expression was observed toward a more endothelial-like phenotype |

BrdU: bromodeoxyuridine; CD34: cluster of differentiation 34; DiI: a fluorescent lipophilic dialkylcarbocyanine dye for labeling cells; GFP: green fluorescent protein; ICC: immunocytochemistry; ICH: immunohistochemistry; K3: keratin 3; K12: keratin 12; Kera: keratocan; Lum: lumican; SSEA4: stage-specific embryonic antigen 4; SP: side population cells (flow cytometry); WB: Western blotting.

**Table 36.4** Characterization of limbal MSC phenotype by flow cytometry.

| Reference | Culture medium | Marker | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | CD29 | CD34 | CD44 | CD45 | CD73 | CD90 | CD105 | CD146 | HLA | |
| | | | | | | | | | | ABC | DR |
| Polisetty *et al.* [108] | MEM/F12/FBS | ✓ | × | | × | | ✓ | ×[a] | | ✓ | × |
| Polisetti *et al.* [109] | DMEM/FBS | | × | ✓ | | | ✓ | | | ✓ | × |
| Ainscough *et al.* [107] | DMEM/FBS | | × | | × | | ✓ | | | | |
| Branch *et al.* [110] | M199/FBS | ✓ | × | ✓ | × | ✓ | ✓ | ✓ | | ✓ | × |
| Bray *et al.* [111] | DMEM/F12/FBS | | × | | × | ✓ | ✓ | ✓ | | | |
| | MesenCult-XF® | | × | | × | ✓ | ✓ | ✓ | | | |
| Garfias *et al.* [112] | DMEM/F12/FBS | ✓ | ✓[a] | | × | ✓ | | ✓ | | | × |
| Bray *et al.* [113] | DMEM/F12/FBS | ✓ | × | ✓ | × | ✓ | ✓ | ✓ | ✓[a] | ✓ | × |
| | MesenCult-XF® | ✓ | × | ✓ | × | ✓ | ✓ | ✓ | ×[a] | ✓ | × |

✓: majority (70–95%) of cells reside within the positively gated fraction; ×: majority (70–5%) of cells reside within the negatively gated fraction.
MEM: minimum essential medium; F12: Ham's F12 medium; FBS: fetal bovine serum; DMEM: Dulbecco's modification of Eagle's medium; M199: medium 199; MesenCult-XF®: commercial serum-free culture system for MSC expansion (StemCell Technologies).
[a] Distinct differences observed between and within studies.

MSCs into corneal cells seems likely to be an essential requirement for their clinical use. This field of research has recently become more complex by reports of MSC-like properties from cultures established from the peripheral corneal stroma – the limbal stroma, the cells of which are known as keratocytes in distinction from the epithelial cells of the cornea, which are known as keratinocytes (Table 36.4). The relationship and relative value of corneal-MSCs compared with traditional sources of MSCs, such as bone marrow, are at present unclear.

After providing a concise overview of corneal structure and function, we will highlight the types of corneal diseases that are likely to benefit from the anti-inflammatory and immunomodulatory properties of MSCs. We will subsequently summarize the evidence supporting the case for MSC-based therapies in the treatment of corneal diseases. Then we will review the literature concerning the keratogenic potential of MSCs. Finally, we will review the more recent literature indicating the presence of MSC-like cells derived from corneal tissue.

## 36.2 Anatomy and physiology of the human cornea

The cornea is the primary refractive element in the visual pathway. It consists of a highly transparent corneal stroma sandwiched between two epithelial tissue layers (Figures 36.1 and 36.2).

The anterior corneal epithelium, like the epidermis of skin, arises, from the ectodermal germ layer of the primitive embryo but forms a nonkeratinized stratified squamous epithelium consisting of between five and seven layers of cells. This epithelium is distinguished from other epithelial tissues in the body by expression of cytokeratin (also known as keratin) 3 and cytokeratin 12 [15]. Renewal of this epithelial layer throughout life occurs by the division and differentiation of epithelial progenitor cells that reside within the peripheral margin of the cornea known as the corneal limbus. More specifically, these so-called corneal stem cells are concentrated deep within epithelial crypts (Figure 36.1D) that reside between undulations of vascularized stromal tissue known as the palisades of Vogt. These are named after Alfred Vogt (1879–1943), a Swiss ophthalmologist.

Immediately posterior to the basement membrane of the corneal epithelium resides an 8–10 μm wide acellular zone of randomly organized extracellular matrix (ECM) components known as Bowman's layer (BL – named after Sir William Bowman (1816–1892), an English physician, anatomist, and ophthalmologist, who discovered it) (Figure 36.1C). While the activities of BL remain unclear, it is interesting to note that the termination point for this structure coincides with the location of the limbal stem cell niche. The adjacent corneal stroma constitutes approximately 90% of the thickness for the entire cornea and contains resident cells known as keratocytes distributed within several layers of highly organized ECM components including collagen, keratocan and lumican. In addition to producing these ECM components, keratocytes are defined by expression of aldehyde dehydrogenase and the cell surface marker CD34 [16]. In some respects, keratocytes are analogous to fibroblasts found within the dermal layer of skin. However, keratocytes are derived from cranial neural crest, as opposed to somatic mesoderm, and form an interconnected syncytium, more akin to the arrangement of osteocytes observed in bone tissue. In normal healthy corneas keratocytes are considered to be relatively quiescent, but corneal damage promotes their conversion into wound repair fibroblasts ($CD34^-$ and $CD90^+$) and myofibroblasts ($CD34^-$, $CD90^+$, α-smooth muscle $actin^+$ (SMA)) typical of stromal cell responses in other tissues.

In addition to keratocytes, the anterior stroma is populated by a dense network of sensory nerve fibers that extend their

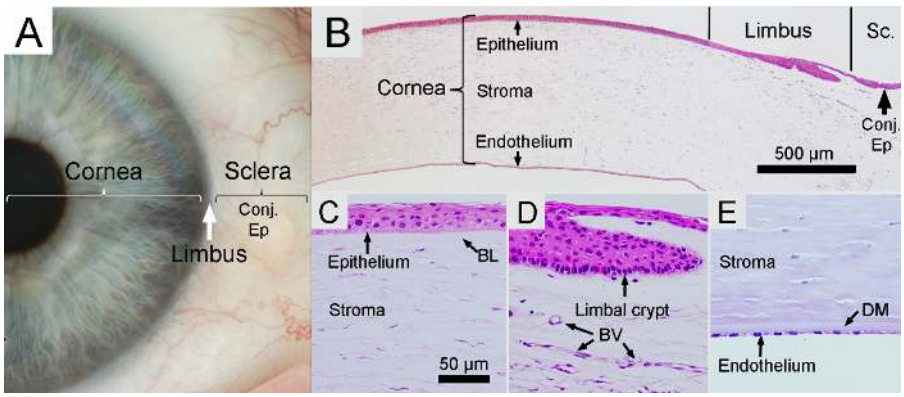



**Figure 36.1** (A) Overview of normal ocular surface anatomy highlighting relative location of the transparent cornea (iris observed in background), corneal limbus, sclera, and conjunctival epithelium (Conj. Ep). (B) Histological section of peripheral cornea (stained with hematoxylin and eosin) illustrating relative location and structure of corneal epithelium, stroma, endothelium, limbus, sclera (Sc.), and conjunctival epithelium (Conj. Ep). (C) Higher power view of corneal epithelium, Bowman's layer (BL), and adjacent stroma. (D) Higher power view of corneal limbus in which a limbal crypt (stem cell niche) and numerous blood vessels (BV) can be observed. (E) Higher power view of posterior cornea illustrating corneal endothelium and Descemet's membrane (DM). The final magnification of parts (D) and (E) is the same as for part (C).

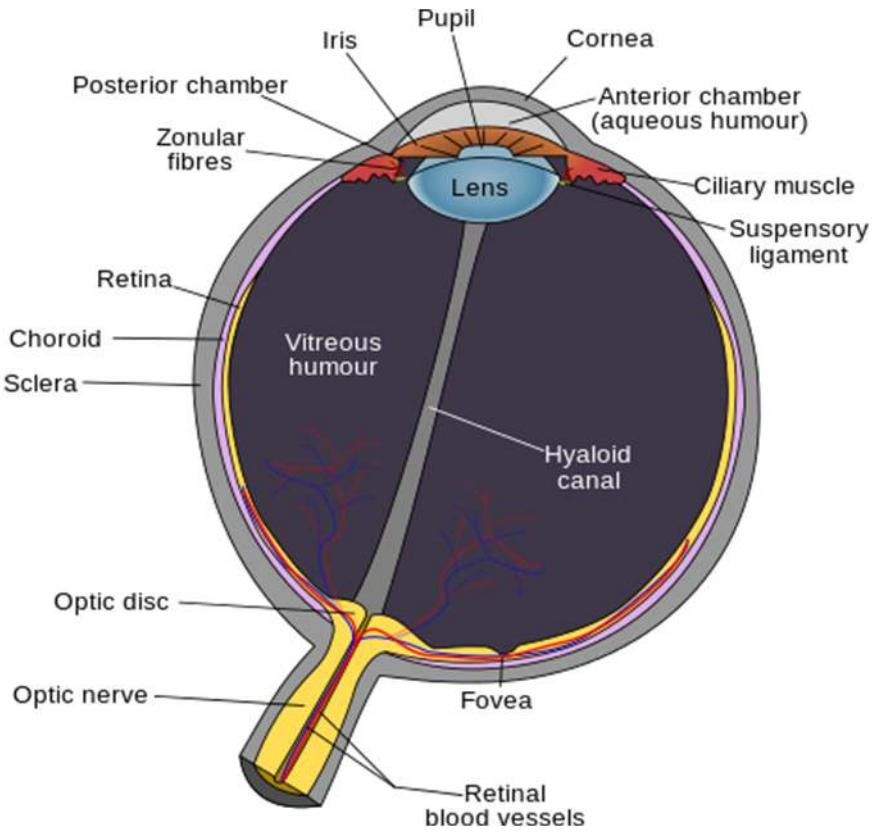



**Figure 36.2** Schematic diagram of the anatomy of the human eye. *Source:* http://commons.wikimedia.org/wiki/File:Schematic_diagram_of_the_human_eye_en.svg#/media/File:Schematic_diagram_of_the_human_eye_en.svg.

processes across BL and into the corneal epithelium. Under normal circumstances, blood vessels and lymphatic vessels are restricted to the corneal limbus, but small numbers of antigen-presenting cells (APCs) have been observed within the corneal stroma. The limited contact with cells of the immune system is often regarded as inferring a form of immune privilege for corneal tissue; but, as will be discussed later, this privilege is far from absolute (Sections 36.5.1 and 36.5.2).

Posterior to the corneal stroma resides a second population of neural-crest-derived cells known as the corneal endothelium (Figure 36.1E). The corneal endothelium, or posterior corneal epithelium, is a single layer of epithelial cells separated from the posterior stroma by a thick basement membrane known as Descemet's membrane (named after the French physician Jean Descemet (1732–1810)). Unlike corneal epithelial cells and stromal cells, corneal endothelial cells display far less capacity for self-renewal, and a gradual decline in the number and density of these cells is observed with age [17]. Loss of corneal endothelial cells through disease or trauma is therefore generally more problematic than the loss of corneal epithelial cells or keratocytes. Phenotypically, the corneal endothelium is generally defined by a collection of nonspecific markers, including N-cadherin, zonula occludens-1 (ZO-1), and sodium/potassium ATPase.

All three layers of the cornea contribute to the optical properties of this tissue. The corneal epithelium, in addition to its protective barrier function, maintains a smooth transparent ocular surface and contributes to the refractive power of the cornea. Transparency of the corneal stroma is essential for visual acuity, and this is enabled through the highly ordered arrangement of keratocytes and ECM components. The hygroscopic composition of ECM components within the corneal stroma encourages the diffusion of water and accompanying solutes from the anterior chamber of the eye. (Hygroscopy is the ability of a substance to attract and hold water molecules from the surrounding environment.) In doing so, however, the optimal spacing of ECM components required for transparency would soon be lost if not for the action of the corneal endothelium. The sodium/potassium ATPase produced by these cells actively transports ions out of the corneal stroma and back into the anterior chamber of the eye. The net movement of ions encourages water to follow by osmosis, and thus corneal transparency is maintained. A decline in number or activity of corneal endothelial cells through disease or trauma may therefore lead to corneal opacity.

## 36.3 Overview of corneal pathology

Diseases of the cornea and ocular surface cover a wide range of conditions, including microbial infections, disorders of the immune system, congenital and metabolic abnormalities,

dysplasia, and malignancies [18]. Moreover, trauma can result in significant pathology, especially when it involves significant loss of limbal epithelial progenitor cells or corneal endothelial cells. The conditions that are most relevant to a discussion of potential MSC-based therapies are those involving chronic inflammation of the ocular surface and those requiring a donor corneal transplant.

### 36.3.1 Ocular surface disease

The corneal epithelium, together with the conjunctival epithelium, tear film, and accessory glands, contributes to maintenance of a smooth and lubricated ocular surface. As stated earlier, the principle role of the corneal epithelium, beyond simply its barrier properties, is to maintain a smooth and transparent structure suited to the refraction of light. Naturally, any challenge to epithelial integrity resulting from biological, physical, or chemical agents can therefore impact significantly on corneal function. In the case of normal wear and tear the corneal epithelium heals itself through the division, differentiation, and migration of cells arising from limbal epithelial progenitor cells. Any disturbance to epithelial homeostasis or tear formation, however, can lead to conditions collectively referred to as ocular surface disease.

Pathologically dry eyes can normally be managed through administration of artificial tears or other treatments. However, people with impaired wound healing responses, such as those with diabetes mellitus, are also prone to nonhealing epithelial defects. Such relatively common forms of ocular surface disease, while debilitating, can be managed reasonably well without the need for surgery or cellular therapies. It remains possible, however, that products derived from MSCs could be beneficial as an adjunct therapy for recurrent epithelial defects, and there is already some anecdotal evidence to support this [19].

Corneal diseases and injuries that extend to the limbus can dramatically reduce the number of epithelial progenitor cells required to sustain the corneal epithelium. In the absence of a corneal epithelium, the surrounding *conjunctival* epithelium extends across the corneal surface. Given the reduced optical properties of the conjunctival epithelium, corneal clarity is reduced. Moreover, the conjunctivalized corneal surface is prone to epithelial defects that, in turn, promote chronic inflammation of the corneal stroma, including scarring and vascularization. Because of the common involvement of limbal dysfunction, conditions associated with a conjunctivalized ocular surface are collectively referred to as limbal stem cell deficiency (LSCD) [20]. Typical causes of LSCD include trauma resulting from alkali burns or surgery to remove limbal tumors. Disorders of the immune system such as Stevens–Johnson syndrome, while relatively rare, can also have devastating effects on the ocular surface, including the limbal stem cell niche. Similarly, the congenital disorder aniridia (absence of the iris), arising from mutations in the *PAX6* gene, obstructs development of several structures within the anterior segment of the eye, including the corneal limbus, thus resulting in pathology consistent with other forms of LSCD, including the overgrowth of conjunctival tissue and chronic inflammation leading to stromal scarring and vascularization. The iris is a thin circular structure responsible for controlling the diameter and size of the pupil, and thus the amount of light reaching the retina (Figure 36.2). The color of the iris gives the eye its color. In optical terms the pupil is the eye's aperture and the iris is the diaphragm that serves as the aperture stop. The iris controls the size of the pupil by its smooth muscles: these are the sphincter muscle (sphincter pupillae), which contracts the pupil in a circular motion, and a set of dilator muscles (dilator pupillae), which pull the iris radially to enlarge the pupil, pulling it in folds.

### 36.3.2 Diseases of the corneal stroma and endothelium

The corneal stroma may be directly affected, as in conditions such as keratoconus, or indirectly affected via diseases of the corneal endothelium that lead to swelling of the corneal stroma.

Keratoconus is a relatively common degenerative disease with an incidence of 1 in 2000 people in Australia and is characterized by gradual thinning and ectasia, or protrusion, of the corneal stroma [21]. While the exact causes of keratoconus are unclear, links have been made to behavioral traits such as eye rubbing. Additionally, genetic mutations have been implicated. Typically, the condition presents in early adulthood and can be managed initially by wearing spectacles. If the condition progresses, hard contact lenses may be required to overcome the high degree of irregular astigmatism (an optical defect in which vision is blurred due to the inability of the optics of the eye to focus a point object into a sharp focused image on the retina).

Advanced cases of keratoconus may require corneal transplantation. A more recent innovation involves removing the corneal epithelium to expose the corneal stroma, followed by chemical crosslinking of stromal collagen fibers. Riboflavin (vitamin $B_2$) has been used as the crosslinking agent. This is activated by exposure to ultraviolet light. Collagen crosslinking can slow or even halt the progression of corneal ectasia.

Examples of endothelial dysfunction fall into two main categories: corneal dystrophies, arising through congenital abnormalities, and reductions in corneal endothelial cell density, brought about through age and trauma. Fuch's dystrophy is a relatively common degenerative disorder (1 in 2000 people) associated with thickening of Descemet's membrane, increased apoptosis of corneal endothelial cells, stromal swelling, and eventually blistering of the ocular surface [22]. While the exact mechanism by which Fuch's dystrophy develops remains somewhat unclear, it displays an autosomal dominant pattern of inheritance and has been linked to missense mutations in the gene for the alpha-2 chain of type VIII collagen. Fuch's dystrophy is generally regarded as a primary defect in endothelial cell function, and thus treatments are based upon targeting this tissue through either medical or surgical techniques. Endothelial dysfunction can also occur as an unintended consequence of intraocular surgery. Historically, endothelial damage has been associated with surgery to treat cataracts; however,

improvements in surgical techniques have markedly reduced the degree of endothelial cell loss. Iatrogenic endothelial dysfunction (e.g., caused by surgery) is generally referred to as bullous keratopathy. Fuch's dystrophy was first described by the Austrian ophthalmologist Ernst Fuchs (1851–1930).

## 36.4 Corneal transplantation and cultivated epithelial autografts

The standard treatment for severe corneal diseases, including advanced keratoconus, Fuch's dystrophy, and bullous keratopathy, is to perform a donor corneal tissue transplant. Historically, corneal transplants involve complete replacement of the entire central cornea from epithelium through to endothelium by a procedure referred to as penetrating keratoplasty (PKP) [1]. In more recent times, however, surgeons have adopted partial-thickness transplantation techniques with the view to more specifically replacing only those areas of the cornea that are diseased [22]. For example, in cases of endothelial dysfunction, a surgeon may attempt to replace only the corneal endothelium along with Descemet's membrane. Alternatively, performing a lamellar graft, where only the anterior portion of the cornea is replaced with donor tissue, may be performed to retain a healthy corneal endothelium. This more conservative approach to corneal transplantation makes sense from the viewpoint of preserving healthy tissue structure. Moreover, the corresponding use of less donor tissue reduces the foreign antigenic load, theoretically reducing the risk of immunological rejection of the graft. Subsequently, the use of PKP has reduced significantly. Irrespective of the surgical technique used, transplants of the central cornea remain the most successful form of solid organ transplant, with most transplants expected to last approximately 10 years in the absence of tissue typing or immunosuppressive therapy [7,23]. Nevertheless, the survival curves for corneal transplants change dramatically when the margin of transplanted tissue is extended to include the limbus (Figure 36.3).

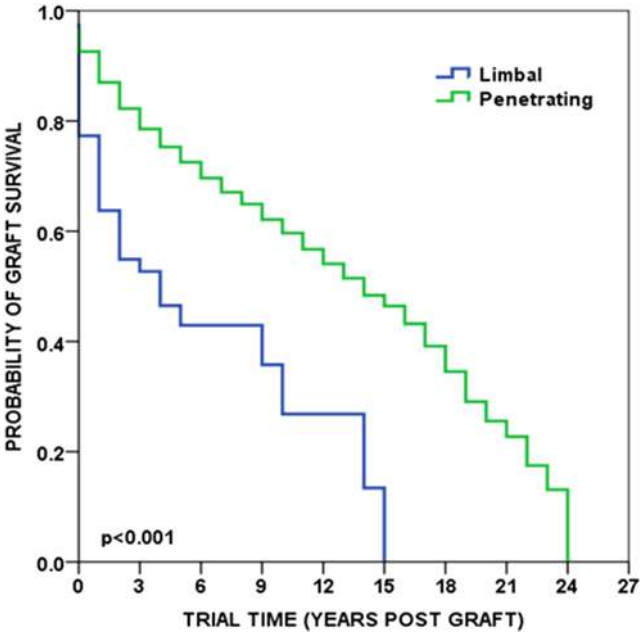



**Figure 36.3** Kaplan–Meier plots displaying the survival of full-thickness transplants of central donor cornea (penetrating keratoplasty) compared with transplants containing donor limbal tissue (log rank statistic = 50.799; df = 1; $P < 0.001$). *Source*: [24] Reprinted with permission from The Australian Corneal Graft Registry, Department of Ophthalmology, Flinders University, Adelaide, South Australia, Australia (taken from The Australian Corneal Graft Registry 2012 Report; KA Williams, MT Lowe, MC Keane, VJ Jones, RS Loh, DJ Coster (eds); 2012 pp. 1–246. URL: http://hdl.handle.net/2328/25859).

Two important points need to be made. First, while donor corneal transplants display relatively high survival rates, they will eventually fail due chronic immunological rejection (Figure 36.4). A failed corneal transplant is therefore a common reason for patients receiving a repeat corneal transplant. Techniques for improving the survival of corneal allografts are a priority area of research. Second, there are alternatives to donor tissue transplantation when diseases and injuries affect the corneal limbus, and especially in the case of LSCD.

Given the failure rate of donor limbal transplants, some surgeons have utilized small autologous transplants of one to two clock hours in shape, typically obtained from the contralateral healthy eye with limbal stem cells to spare [25]. Nevertheless, this technique risks creating localized stem cell deficiency in an otherwise healthy eye. As an alternative, therefore, many surgeons have switched to removing a much smaller piece of healthy limbal tissue from which a sheet of new corneal epithelium could be grown *ex vivo* before transplantation on to the surface of the injured eye [4,5]. Limbal epithelial cells and their progeny are first grown using a technique initially established for the cultivation of epidermal skin grafts developed by Rheinwald and Green in 1975 [26]. This technique relies on the use of growth-arrested mesenchymal cells in the form of embryonic murine 3T3 fibroblasts as a source of ECM proteins and growth factors to encourage the establishment of epithelial colonies from enzymatically dispersed patient tissue. More commonly, however, explants of limbal tissue have been grown intact while attached to a sheet of devitalized donor amniotic membrane [3,27]. In either case, some form of substrate is required to support the cultured epithelial sheet during its application to the ocular surface. A number of substrates have been successfully used, including donor amniotic membrane, fibrin glue, collagen membranes, and contact lenses. In many cases, surgeons have been able to improve the smoothness and transparency of severely injured corneas, with the best results often being obtained in conjunction with the use of PKP to remove stromal scarring at a later date [28]. Importantly, however, cultured sheets of limbal epithelial cells are not a permanent solution since long-term success relies on any progenitor cells remaining at the time of treatment and reestablishing themselves within a limbal stem niche. The treatment of severe corneal

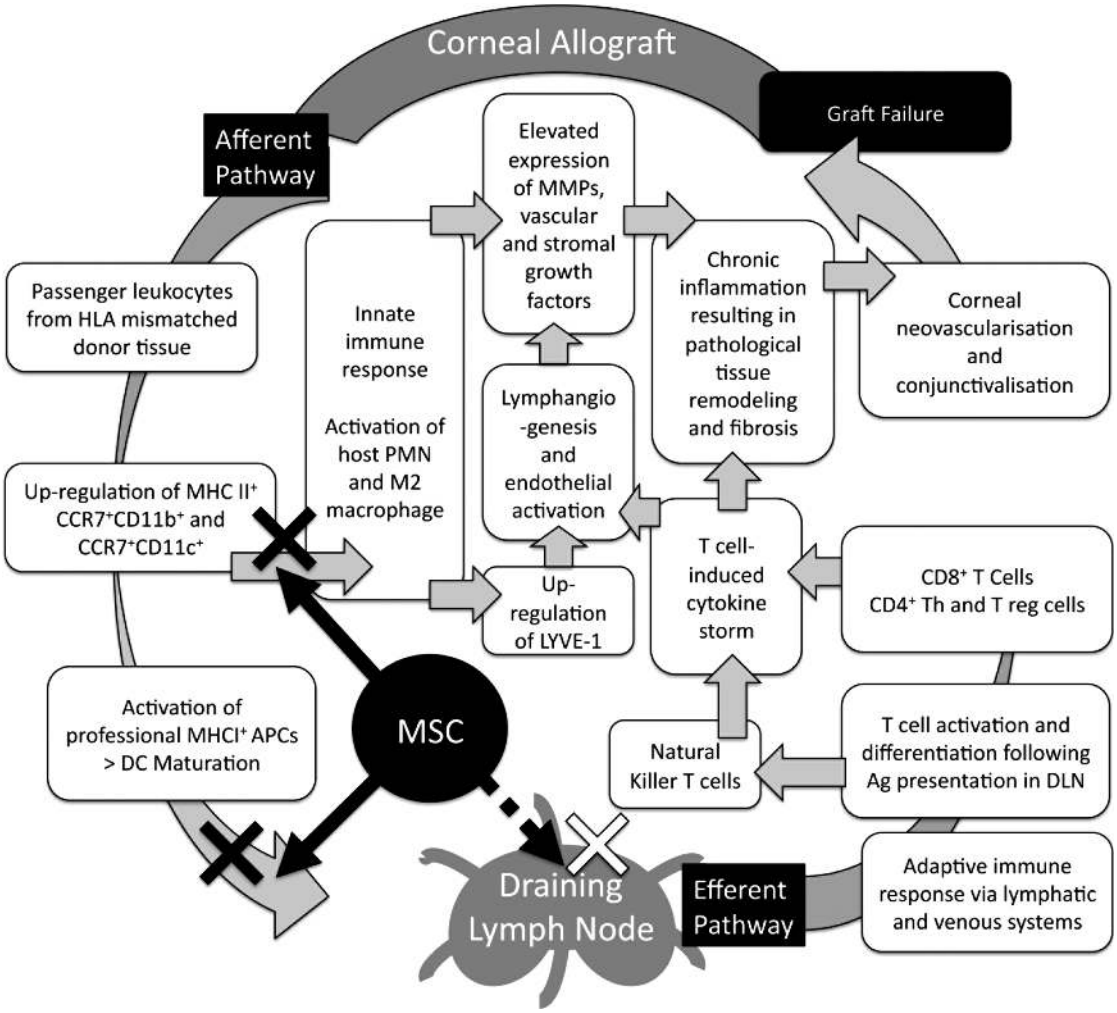



**Figure 36.4** Schematic illustrating the immune reflex arc involved with donor corneal transplant (corneal allograft) rejection. Potential sites of direct (full black arrows leading to black cross) and indirect (broken arrow leading to white cross) inhibitory actions of perioperative MSC-based therapies are illustrated.

disease could be enhanced significantly by finding ways to improve the survival of donor tissue transplants, especially those containing limbal tissue. Adjunct therapies based upon the use of MSCs therefore provide an attractive topic for investigation.

## 36.5 Evidence for mesenchymal stromal cells as modulators of corneal disease

Before considering a potential role for MSCs in treating corneal disease, it is helpful to explore the immunology of the cornea and the immune response to donor corneal transplants.

### 36.5.1 Immunology of the cornea

Both innate and adaptive immune systems play a role in preventing infection and repairing wounds to the corneal surface, although such mechanisms do not directly involve any lymphoid cells. Tear formation, corneal nerve reflexes, transforming growth factor-β1 (TGF-β1) production from keratocytes, and expression of interleukin (IL)-1α by epithelial cells, as well as interferons and the complement system, all operate to ensure that the epithelium remains impervious to external damage. The cells of the adaptive immune system are active within the corneal stroma and adjoining regions in the anterior segment of the eye. They respond to specific events such as infection, but they are also mediators of ongoing inflammatory damage and corneal transplant rejection. T cell precursors and some APCs residing in the anterior chamber are capable of responding to foreign antigens and initiating focal and systemic inflammatory responses. While there are no histamine-producing cells in the normal cornea because of its lack of a blood supply, it appears that there is a heterogeneous network of bone-marrow-derived cells that can be stimulated to function as APCs in certain conditions [29]. This unique population of both immature and mature cells includes epithelial Langerhans cells and anterior stromal dendritic cells (DCs), which are present in the peripheral cornea and to a lesser extent in the central

cornea [30]. Following an inflammatory insult, this population of DCs matures and in the process expresses major histocompatibility complex (MHC) class II antigens as well as the B7 antigens CD80, and CD86 and CD40 costimulatory molecules. This leads to downstream activation of T helper cells [31]. The corneal endothelial cells also contribute through weak expression of MHC class I antigens as well as Fas ligand (CD95L), resulting in apoptosis of lymphocytes [32].

Given its limited immune interaction the cornea is generally defined as being immune privileged, although this term is relative as, following major trauma or infection, bone-marrow-derived cells mature and infiltrate the cornea, producing keratitis and, in more chronic cases, diffuse vascularization and corneal fibrosis. Even in minor events, once the inflammation is mitigated, the cornea continues to retain a greater population of resident leukocytes than normal [33]. In part this is due to the involvement and proximity of the conjunctiva and the abundance of phagocytes and lymphocytes that circulate through the microvasculature of these tissue structures. The conjunctiva provides further immunological support to the cornea. However, in cases of severe focal trauma, it also provides a reservoir of cells from the innate immune system that stimulate angiogenesis, granuloma formation, fibrosis, and pathological conjunctivalization, creating a chronic inflammatory milieu that protects the corneal stroma without resolving the initial injury [34].

### 36.5.2 Immunology of corneal transplantation

For more than a century human corneal transplant procedures have been successfully performed to restore the corneal epithelial surface and reverse the optical disability. Typically, there is a 90% or better rate of graft survival within the first 12 months after corneal allograft surgery. This is quite remarkable compared with outcomes of other solid organ transplantation, such as lung transplants or pancreatic transplants. However, over the course of the next 15 years after a corneal transplant the failure rate is much higher (Figure 36.3), with as many as 55% of allografts failing [7]. It is of interest that the rate of primary graft failure appears to be directly proportional to the degree of focal inflammation and vascularization of the cornea prior to transplantation, even after accounting for confounding variables such as surgical approach, operator, comorbidities, and preexisting risk factors [7,35,36].

There are many reasons for graft dysfunction, including infection, inadequate immunosuppressive therapy regimens, poor human leukocyte antigen (HLA) matching, and most importantly the efferent immune response that is an irreversible reaction refractory to corticosteroid and other immune-modulating treatments (Figure 36.4). It is this focal host-versus-graft response that is the major cause of corneal allograft rejection [6].

In considering the pathophysiological mechanisms that lead to graft rejection, it is pertinent to note that there are two anatomically separate populations of APCs located in the iris and trabecular meshwork and in the conjunctiva and cornea [37]. Macrophages and MHC class II$^+$ DCs of the first group are well positioned to absorb soluble antigen shed from the posterior endothelial layer of the graft, and iris phagocytes are known to play a role in activating T cells during secondary responses [38]. MHC class II DCs of the conjunctiva and cornea appear to be specific to the local environment in which they reside in terms of phenotype and density [39]. Owing to the immunoprivileged nature of this anatomical region these cells do not normally encounter foreign antigens that stimulate the reactive T cell responses leading to a rejection process [33,40,41]. This is particularly noteworthy given that it has been reported in a preclinical murine model that donor cornea is a source of "passenger" leukocytes that migrate into cervical draining lymph nodes (DNLs) and start expressing MHC class II antigen after surgical implantation [33,41].

In considering the mechanisms of chronic rejection following corneal transplantation it is worth pondering how this process occurs given that the normal cornea is avascular and without a lymphatic drainage system. It is noteworthy that lymphatic vessels that express lymphatic vessel endothelial receptor 1 (LYVE-1) can invade an inflamed cornea and stimulate the process of neovascularization and lymphangiogenesis [42,43]. LYVE-1 is a CD44 homolog expressed by lymphatic endothelial cells. However, Xu *et al.* localized this marker to nonendothelial murine cells in the sclera, choroid, and iris where cell surface marker labeling was indicative of CD11b$^+$ F4/F80$^-$ bone marrow cells, CD11b$^+$ F4/F80$^+$ macrophages, and CD11b$^+$ F4/F80$^-$ macrophages [44]. The highest densities of LYVE-1$^+$ cells were observed in the limbus and conjunctiva. All LYVE-1$^+$ cells in the retina were CD11b$^+$ F4/80$^+$ macrophages. Cells in the limbus and the iris root also expressed the stem cell antigen Sca-1, suggesting that they are hematopoietic lymphatic vessel progenitor cells. Given that LYVE-1$^+$ CD11b$^+$ F4/F80$^+$ macrophages are present in the normal eye, they should be able to participate in the stimulus for lymphangiogenesis in response to any uncontrolled inflammatory challenge.

Immune privilege can be reversed due to the demonstrated presence of APCs in an avascular cornea prior to transplantation as well as by donor-derived APCs resident in the graft tissue at the time of surgery. The degree of major and minor histocompatibility matching between the corneal transplant donor and the corneal transplant recipient will also influence the risk of rejection. It has been suggested that the graft tissue is degraded overtime as a result of the inflammatory sequelae generated by histoincompatibility between donor and recipient, corneal angiogenesis, and lymphangiogenesis and the apoptosis that results from cytotoxic T-cell- and natural killer (NK)-cell-mediated mechanisms [45–48].

While the first corneal transplant was performed in 1906 and the first renal transplant in 1960, long-term improvements in clinical outcomes over the last 56 years in solid organ vascularized transplants have surpassed those in corneal transplantation [23]. This is largely a result of the improved immune suppression regimens used after vascularized solid organ transplantation. However, the location of the cornea makes it all the more

amenable to modern therapeutic strategies that utilize bioengineered antibodies and novel regenerative medicine approaches, such as mesenchymal stem/stromal cell administration.

### 36.5.3 Immunomodulatory properties of mesenchymal stromal cells

MSC administration is currently the subject of intense scientific investigation and clinical trials in an attempt to assess their safety and efficacy as both a novel immunomodulatory treatment and a potential wound healing and allograft alternative. These cells are relatively nonimmunogenic due to their lack of cell surface expression of CD40, CD80 and CD86 co-stimulatory molecules, and MHC class II molecules [49,50]. They are also actively suppressive of T cell alloproliferation as well as suppression of B cell, DC, and NK cell activity.

When MSCs are differentiated *in vitro* to the mesodermal adipogenic, osteogenic, and chrondrogenic lineages there is a slight increase in MHC class II expression, but not of the co-stimulatory molecules, indicating that these cells are capable of inducing tolerance and specifically T cell anergy [51–53].

MSCs also have an inhibitory effect on the expression of the proinflammatory cytokines TNF-α and IL-12 during DC differentiation [54,55]. In addition, MSCs can prevent peripheral blood mononuclear cells from differentiating into DCs [56]. When co-cultured with lymphocytes T cell growth kinetics were suppressed even after the addition of interferon (IFN)-γ [57]. MSCs have been reported to halve the expression of IFN-γ during T-cell differentiation and increase the production of IL-4 during type 2 helper T cell differentiation [54]. They can prevent cytotoxic T cell activation when added early to a mixed lymphocyte population [58].

MSCs are also capable of inhibiting the cell cycling behavior and chemotactic properties of B cells and can prevent their terminal differentiation into plasmacytes [59]. Factors in culture medium derived from MSCs inhibited immunoglobulin (Ig)G1 and IgM production in a murine model immunized with T-cell-dependent and -independent antigens [60].

MSCs are highly responsive to the endocrine and paracrine signaling cues within an inflammatory environment. The level of factors such as IFN-γ at low levels may induce MSCs to behave as APCs and promote the generation of cytotoxic $CD8^+$ T cells or, at higher concentrations, mediate immunosuppressive activities [61,62].

The local inflammatory environment is key in determining the behavior of MSCs. Van Poll *et al.* showed that the intravenous administration of MSC culture medium resulted in a 90% decrease in hepatic apoptosis and increased survival outcomes if administered before the systemic inflammatory response syndrome transitioned to multiple organ failure in a murine model in which acute liver failure was induced by D-galactosamine [63].

Other additional immunomodulatory mechanisms associated with MSCs include the ability to synergistically recruit and support anti-inflammatory processes instigated by regulatory T cells (Tregs). MSCs increased the numbers of Tregs when cultured with $CD4^+$ T cells [54]. Human adipocyte-derived MSCs can upregulate the anti-inflammatory mediator IL-10 as well as the numbers of $Foxp3^+$ $CD4^+$ $CD25^+$ Tregs [64]. MSCs play a role in directing the behavior of monocyte/macrophages populations in sepsis, where removal of these innate immune system cells impaired the ability of MSCs to attenuate systemic inflammatory and infectious processes [65].

The ability of MSCs to activate immunosuppressive networks and mediate adaptive and innate immune tolerance has led to the view that MSCs are an essential part of setting the background of tissue architecture and might reasonably be regarded as local stromal coordinators by ameliorating excessive focal and systemic immune responses [66]. It is for this reason that MSC administration has been explored as a potential treatment for both congenital and acquired causes of LSCD.

### 36.5.4 Mesenchymal stromal cells as modulators of corneal wound healing and tissue regeneration

MSCs have been found to positively influence the *in vitro* growth of corneal epithelial cells [67] and corneal endothelial cells [68]. It is reasonable, therefore, to assume that they could facilitate corneal wound healing *in vivo*. MSCs are being used clinically to treat persistent epithelial defects in patients refractory to conventional treatment [19]. The effect of MSCs on corneal wound healing in rodent models are presented in Table 36.1.

The majority of preclinical studies have examined the effects of MSC administration following chemical (alkali or alcohol) removal of the corneal–limbal epithelium [11,14,69,71–76]. Routes of MSC administration have included topical application as a cell suspension [69,76], intravenous injection [11], intraperitoneal injection [11], or subconjunctival injection [73,75].

MSCs have also been applied in conjunction with amniotic membrane [14,71,76] or synthetic scaffolds [70,74]. In these studies MSC administration generally led to marked clinical improvements, including reduced inflammation, reduced neovascularization, and improved re-epithelialization. The mechanism of MSC action has been linked in some cases to a reduction in production of proinflammatory cytokines [70,72–74].

Following intravenous or intraperitoneal injection of $1 \times 10^7$ human MSCs, Roddy *et al.* [72] determined that approximately 0.0001% of the cells were localized to the corneal tissue at 1 day or 3 days after the corneal injury and removal of limbal stem cells. While there was little evidence of engraftment, there was a significant reduction in the levels of focal inflammation and opacity within the cornea. MSC expression of the anti-inflammatory mediator TSG-6 was increased following corneal epithelial cell insult in *in vitro* experiments. These results were later validated in a TSG-6 small interfering RNA knockdown model. Together, these findings suggest that MSC administration activates a negative feedback loop to dampen inflammation and reduce leukocyte recruitment in tissue adjacent to the cornea.

The antiangiogenic and anti-inflammatory effects of MSCs in wound healing were explored by Oh *et al.* [69], where absolute ethanol was applied to denude the corneal surface in a rodent model. Animals were randomized to receive one of three MSC interventions or a control administered topically to the eye. The interventions were fresh culture medium (as control), MSC-conditioned medium (MSC-CM) once only for 2 h, MSC-CM once daily for 3 days (MSC-CM multiple), and direct MSC application. All treatment groups had evidence of peripheral neovascularization in conjunction with corneal opacity by 3 weeks after the injury, but there were significant differences in inflammatory cell infiltrates in the MSC-CM multiple and direct MSC application groups compared with the control group. The proinflammatory markers IL-2 and IFN-γ levels were decreased in the MSC-CM multiple and direct MSC treatment groups. The anti-inflammatory markers TGF-β1 and IL-10 were increased and TSP-1, a surrogate anti-angiogenic marker, was upregulated in the direct MSC and MSC-CM multiple cohorts. PKH26-labeled cells indicated that re-epithelialization occurred above the MSC layers in the direct MSC treatment group. Topical MSC application was thus the most efficacious approach in reducing the overall inflammatory burden 3 weeks after injury, presumably by the constant active expression of cytokines and growth factors that ameliorated chronic inflammation and potentially activated the function of remnant progenitor cells in the limbal stem cell niche.

Using an alkali burn murine model Javorkova *et al.* demonstrated that systemic administration of bone-marrow-derived MSCs homed to regional tissue damage and migrated preferentially to the eye that had been injured [78]. MSCs that had been pretreated with IFN-γ were the most effective at reducing the proinflammatory mediators IL-1α, IL-6 and nitric oxide during the first 24 h after the injury. Using sodium hydroxide to degrade the ocular surface in a rat model, it was observed that expression of the proinflammatory IL-17 was reduced in $CD3^+$ $CD4^-$ $CD8^-$ (T cells), $CD3^+$ $CD4^+$ $CD8^-$ (helper T cells), and $CD3^+$ $CD4^-$ $CD8^+$ (cytotoxic T cells) in the draining cervical lymph nodes after intraperitoneal injection of MSCs [79]. IL-17 secretion in double-negative T cells from the cornea was completely suppressed after MSC administration.

### 36.5.5 Mesenchymal stromal cells as modulators of corneal transplantation

In four of five separate rodent models investigating the changes in graft survival and lymphocyte profiles, it was found that intravenous or topical administration of MSCs resulted in significant improvements in corneal graft survival time as well decreasing the activation of pathological T cell populations and the expression of CCR7 from APCs (Table 36.2). Jia *et al.* [81] developed an MHC-mismatched rat corneal allograft model using Wistar rats as donors and Lewis rats as recipients in which bone-marrow-derived MSCs were derived from the Wistar corneal donor population. The recipient group randomly received either MSC administration alone or MSCs in addition to cyclosporine (CSP) as immune suppression. MSC administration was commenced 3 days prior to corneal surgery and continued for 2 days after grafting. CSP was administered for up to 9 days postoperatively at doses that ranged from 1 to 10 mg/kg.

The low-dose CSP/MSC group had increased graft rejection. However, when 2 mg/kg of CSP was combined with MSCs there appeared to be a synergistic response where graft survival was better than either treatment alone. At doses greater than 2 mg/kg the potential effect of MSC administration was obscured by the CSP treatment. This study highlights the importance of drug–MSC interactions and the need to consider dose-dependent effects when determining no observed adverse effect levels in preparation for phase I trials.

With the objective of investigating the effect of bone-marrow-derived MSCs on allosensitization following corneal allografting from C57BL/6 donors (MHC haplotype $H2^b$) to BALB/c recipients (MHC haplotype $H2^d$), Omoto *et al.* [84] identified that systemic administration of MSCs resulted in homing of $GFP^+$ MSCs to the ipsilateral (damaged) cornea and conjunctiva and DNLs. MSC administration resulted in a significant decrease in $CD11c^+$/MHC class $II^+$ mature DCs both in the corneal graft and the DNLs. At 14 days posttransplant, allosensitized type 1 helper T cells were substantially reduced both in the ipsilateral DNLs and in the graft, indicating that MSC administration can be used to modulate alloimmunity in high- and low-risk corneal grafting. In a follow-up study in which MSC administration was administered once only postoperatively or immediately after surgery and 7 days postoperatively, graft survival was more than twice as high in the latter compared with the control group. These findings suggest that MSCs can be used as a first-line or second-line immunomodulatory agent.

In a similar study by Lee *et al.* [83], specific attention was placed on the cytoplasmic expression levels of CCR7, a molecule critical in the migration of lymphocytes and DCs across the high endothelial venules in secondary lymphoid organs. Corneal transplantation was performed using C57BL/6 mice and BALB/c mice in which each animal was randomized to be either a donor or recipient for syngeneic or allogeneic procedures. Human bone-marrow-derived MSCs were infused preoperatively on day 0 or preoperatively as well as postoperatively on day 1. Follow-up was assessed at 7 and 28 days depending on randomization processes and were nominally referred to as early and late rejection respectively. $CD4^+$ T cells increased in allogeneic recipients by day 7 and $CD8^+$ T cells had expanded by day 28 compared with the syngeneic recipients, in which there was no change in these T cell subpopulations within cervical lymph nodes. To investigate the effect of human MSC administration, the numbers of CCR7-labeled $CD11c^+$ corneal epithelial DCs and $CD11b^+$ corneal stromal DCs were assessed using flow cytometry. MSCs were administered either postoperatively or preoperatively (day −1) and postoperatively (day 0). The second approach proved more successful in suppressing allograft rejection and ameliorating the adaptive immune response in which lower numbers of $CCR7^+$ DCs were detected. A single administration of intravenous MSCs

appeared to have no significant effect on the recipients' immune response following transplantation. While MSC administration did not completely inhibit MHC class $II^+$ $CCR7^+$ $CD11b^+$ from infiltrating the cornea, the number of these cells was significantly reduced compared with animals that received a corneal allograft and no MSCs.

The topical application of rat MSCs was evaluated in a preclinical trial by Oh *et al.* [80]. Using a pig-to-rat xenogeneic transplant model, all porcine corneal grafts had failed within 12 days of transplantation. There was no difference in graft survival time between those that received topical rat MSCs and those that did not. Histological examination of the grafts showed a massive infiltrate of neutrophils and macrophages as well as $CD8^+$ and NK T cells. The levels of IL-6 and IL-10 were significantly elevated in the MSC-treated graft tissue.

Subsequently, the same group tested the efficacy of human MSCs administered intravenously both pre- and postoperatively at a dose of $1 \times 10^6$ in a murine allogeneic corneal transplant model [82]. Only a small fraction of the administered cells was found in the cornea, with the majority residing in the lungs, as is normal after intravenous administration of MSCs. There was a significant reduction in the number of APCs in the cornea and DNLs in the allogeneic group compared with the syngeneic control group. The proinflammatory cytokines IL-2 and IFN-γ, as well as the $CD8^+$ cell effector molecules granzyme A, granzyme B, and perforin, were all decreased in MSC recipients. The human MSC administration was noted to significantly increase expression levels of TSG-6, with a 113.8-fold change reported. Using knockout mice for TSG-6, as well as intravenous administration of recombinant TSG-6, they were able to confirm the critical role of this downmodulator of inflammation in this model.

In a study by Treacy *et al.* [85], the effect of pretreating MSCs with IFN-γ was compared with nonpretreated syngeneic, allogeneic, and third-party MSCs. All MSCs were administered systemically via the tail vein at 7 days pretransplant and on the day of the procedure using a dose of $1 \times 10^6$ MSCs on each occasion. Graft opacity was used as the primary endpoint. The allogeneic (Dark Agouti) and third-party (Wistar-Furth) MSCs prolonged corneal allograft survival the longest in the Lewis rat recipients. Cell profiling and gene expression studies suggested a reduction in NK T cells, APCs, and $CD4^+$ $CD25^+$ Tregs as well as IL-1β mRNA levels in the recipients of allogeneic MSCs compared with recipients of syngeneic and IFN-γ-pretreated MSCs. MSCs were also found in the spleen, which had a significantly greater number of the $Foxp3^+$ $CD4^+$ $CD25^+$ Tregs in the allogeneic-MSC-treated cohort compared with the syngeneic MSC recipients. Interestingly, significantly elevated levels of IgG1 and IgG2 antibodies were generated against the allogeneic MSCs, but this did not result in a decrease in graft transparency or dysfunction.

It is clear from the studies just described that MSCs mediate their effects in corneal allografting by downmodulation of proinflammatory factors through an influence on both cells and proteins of the innate and adaptive immune systems, often by secreting anti-inflammatory proteins such as TSG-6 or tolerogenic proteins such as IL-10. In effect, they are unique circulating micro-pharmacies.

## 36.6 Evidence for mesenchymal stromal cells as a source of new corneal cells

One of the first papers to investigate the keratogenic potential of MSCs noted that MSCs derived from human bone marrow survived and formed an epithelial-like structure when applied *in vivo* to the ocular surface of wounded rat eyes [86]. Immunohistochemistry, however, using a monoclonal antibody raised against corneal epithelial cells that detects keratin 3 that also detects keratin 2p/76 (mab AE5) showed no evidence of staining. Negative results were also obtained using a pan-cytokeratin antibody, indicating that no epithelial cells of any type had been derived from the implanted bone marrow MSCs. Critically, these negative results were validated using positive controls and the MSC origin of the cells studied was confirmed by immunostaining for human nuclear antigen. On the basis of this negative result, it might have been expected that interest in the keratogenic potential of MSCs might have waned, but many papers have appeared over the subsequent years, some of which contain positive evidence of MSC differentiation into cornea-like cells (Table 36.3).

### 36.6.1 Mesenchymal stromal cell differentiation into corneal epithelium

While cultivated epithelial autografts have become a widely used method for ocular surface reconstruction, the sensitive location and limited supply of a patient's own corneal epithelial progenitor cells has prompted investigation of alternative sources of corneal epithelial progenitor cells. Mesenchymal cells are perhaps an odd choice for epithelial tissue repair, but mesenchymal-to-epithelial transition occurs during embryonic development. An example is neural crest differentiation into corneal endothelial cells. Thus, it is conceivable that a mesenchymal progenitor cell could perhaps be encouraged under the right circumstances to adopt an epithelial phenotype. In the case of the corneal epithelium, however, such a transition would presumably initially require reprogramming of MSCs to an ectodermal lineage, followed by an ocular tissue pathway of differentiation (defined by expression of Pax-6), and finally differentiation into corneal epithelial cells expressing keratin (K) 3 and K12. Interestingly, Pax-6 expression, which is also required for pancreatic islet development, has been reported in cultures of insulin-secreting cells derived from adipose tissue MSCs [96]. No studies, however, have examined Pax-6 expression by MSC-derived from any noncorneal tissue within the context of corneal differentiation. The case for MSC differentiation into corneal epithelial cells has so far been based largely on looking for evidence of K3/K12 expression on corneal cells either *in vitro* or *in vivo*. These studies

have often used the AE5 monoclonal antibody raised against corneal epithelial cells [15], but this antibody also reacts toward cytokeratin 2p/76, a keratin found in palate tissue. Additionally, a number of prior studies have relied solely on immunohistochemical staining [88,90]. Several reports of positive expression have not been validated because of lack of adequate controls [88,97–99]. Moreover, in the case of studies performed *in vivo*, the provenance of reported "corneal cells" is often unclear due to the fact that no marker was used to track the fate of administered MSCs [76,90,98,99]. Despite these shortcomings, several groups have reported a degree of K3 or K12 expression that has been validated through tracking of administered cells and/or use of appropriate experimental controls (Table 36.3) [14,71,87–91]. In most studies the degree of corneal-specific keratin expression was substantially less than that expressed by control samples of corneal epithelial cells [89,91] or noted to reside within only a small subpopulation of MSC-derived cells [14,88,90]. The ability of MSCs to generate corneal epithelial cells therefore remains inconclusive.

### 36.6.2 Mesenchymal stromal cell differentiation into keratocytes

Given their mesenchymal origin (cranial neural crest) and phenotype, keratocytes are perhaps the most likely candidate cell for MSC differentiation in the cornea. Reports of endogenous bone marrow MSC recruitment to injured corneal stroma have led to speculation that MSCs might naturally provide a source of cells for stromal repair. As with the case for studies of epitheliogenesis, prior studies of MSC differentiation into keratocytes have also often been hampered by inadequate use of appropriate cell phenotype markers [11,100] or experimental controls [101,102].

Three studies, however, have produced positive evidence of MSC conversion into keratocyte-like cells that have been appropriately validated. A study by Du *et al.* [92] reported positive evidence of keratocan expression confirmed by Western blotting, RT-PCR, and RT-qPCR when the side population obtained by flow cytometry of adipose-tissue-derived MSCs was grown under conditions designed to promote keratocyte differentiation. Stronger evidence was presented in two studies by Liu and colleagues [13,93] using two null mutant animal models. In the first study, human MSCs derived from umbilical cord were labeled with DiI and injected into the corneas of lumican deficient ($Lum^{-/-}$) and keratocan deficient ($Kera^{-/-}$) mice. As noted earlier (Table 36.1), these treatments led to improvements in corneal structure and function; but more importantly from a cell fate perspective, the implanted cells adopted a keratocyte-like morphology and produced keratocan and lumican, which was confirmed by Western blotting. The second study by this group used MSCs derived from mouse bone marrow and implanted into $Kera^{-/-}$ recipients. The results essentially produced similar results. Given the absence of endogenous keratocan in both studies, the subsequent discovery of this corneal ECM molecule following injection of MSCs is consistent with the theory that MSCs are capable of keratocyte differentiation.

### 36.6.3 Mesenchymal stromal cell differentiation into corneal endothelium

Liu and Zhao [94] reported reduced stromal edema when autologous, BrdU-labeled bone marrow MSCs were implanted onto the posterior surface of rabbit corneas *in vivo*. Survival of the implanted cells was later confirmed by immunostaining for BrdU, but traditional markers of endothelial function, such as ZO-1 and sodium–potassium ATPase, were not examined. The apparent reduction in edema suggests that at least implanted MSCs might provide a useful surrogate for corneal endothelial cells.

A subsequent study by Joyce *et al.* [95] utilizing GFP-labeled MSCs derived from human umbilical cord blood provided a detailed analysis of MSC fate when they were applied to wounded corneal endothelium in organ culture. The effect of growth medium derived from lens epithelial cell cultures was also examined. While these authors detected expression of ZO-1 and N-cadherin in cultures of control MSCs, both proteins became more distributed to cell boundaries in response to CM. A microarray analysis of over 250 genes indicated a shift in MSC phenotype toward that of corneal endothelial cells. Incorporation of MSCs into organ cultures of corneal endothelium appeared to be facilitated by attachment to wounded endothelial cells rather than to exposed sections of Descemet's membrane. These findings support the theory of "licensing," whereby MSCs are activated by local signals such as those produced by damaged cells. Further studies are clearly required to determine the potential of MSCs to produce corneal endothelial cells.

## 36.7 The biology of cornea-derived mesenchymal stromal cells

Following the discovery of epithelial progenitor cells in the corneal limbus and their successful clinical application, speculation began about the potential presence of progenitor cells for other corneal cell types, including corneal stromal cells [103–105]. These studies were motivated in part by the fact that cultivated epithelial autografts, while useful, do not contain the cellular elements required to repair the corneal stroma. Additionally, there has been interest in replacing embryonic murine 3T3 cells with a human mesenchymal cell equivalent, as feeder cells for the *ex vivo* expansion of limbal epithelial progenitor cells.

The foundation for our understanding of corneal stromal cell biology is derived from the work of Elizabeth Fini [16]. She and her colleagues described a population of $CD34^+$ keratocytes, which, upon wounding of the cornea, developed into $CD90^+$ wound repair fibroblasts and myofibroblasts expressing both CD90 and α-SMA. Thus, the $CD34^+$ keratocyte could be considered to represent the endogenous stromal progenitor cell population, with expression of CD90 perhaps representing their immediate progeny of transient amplifying cells. Since the peripheral edge of the corneal stroma extends a short distance (0.5 mm) into the deep limbus, it is likely that a similar pattern of

cellular differentiation occurs in this region. Nevertheless, the superficial limbal stroma contains a variety of additional cell types, including melanocytes, APCs, vascular endothelial cells, pericytes, and glial cells (Schwann cells) [106]. We grew cells from human limbal stroma using Dulbecco's modification of Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and analyzed the resulting cultures by flow cytometry using a panel of antibodies designed to detect a broad range of cell types, including those of corneal origin ($CD34^+$, $CD90^+$, $\alpha$-$SMA^+$), myeloid origin ($CD45^+$), glial origin ($CD271^+$), and vascular/pericyte origin ($CD141^+$, $\alpha$-$SMA^+$). Comparison cultures were established from central corneal stroma, as well as scleral stroma sampled a few millimeters beyond the limbus [107]. Despite the distinct regional differences in tissues acquired, greater than 95% of cells in all cultures expressed CD90 with negligible levels of other cell surface markers. A gradient of $\alpha$-SMA expression was observed ranging from approximately 10% of cells in corneal cultures to up to 80% in cultures established from scleral stroma. Distinct differences were also noted in the ability of cultures established from each region to serve as a feeder layer for limbal epithelial cells, with limbal stromal cells being judged on the number and size of epithelial colonies generated in colony-forming assays. Parallel cultures established from scleral tissue, only a few millimeters away from the limbus, failed to support epithelial growth beyond that seen in the absence of feeder cells.

These findings suggested that cultures established from the limbal stroma are remarkably homogeneous $CD90^+$ fibroblasts and could be used clinically as feeder cells for *ex vivo* expansion of limbal epithelial progenitor cells. The inherent stem cell properties of cells isolated from the corneal and limbal stroma were also becoming understood at this time, with some studies using stem cell markers and culture conditions used successfully for other tissues [104,105]. A different approach, however, was taken by Polisetty *et al.*, who reported that cultures established from the limbal stroma had similar properties to MSC cultures established from bone marrow [108]. This seminal work led to a series of recent publications exploring the phenotype and properties of limbal stromal cell cultures through the prism of MSC biology [109–114] (Table 36.4).

The premise for the initial study by Polisetty *et al.* [108] was that spindle-shaped cells observed as contaminants in cultures of limbal epithelial cells might share phenotypic and differentiation characteristics with bone-marrow-derived MSCs. As summarized in Table 36.4, there was expression of several characteristic MSC cell surface markers, including CD29, CD90, and HLA-ABC, as well as lack of expression of CD34, CD45, and HLA-DR – again the same as for bone marrow MSCs. A subsequent study by this group [109] added CD44 as a further positive MSC marker to this list, but other characteristic MSC markers such as CD105 did not feature prominently in this group's analysis [108]. A subsequent study by Branch *et al.* [110], however, using a panel of MSC markers recommended by the International Society for Cellular Therapies, confirmed that limbal stromal cell cultures established in serum-supplemented growth medium predominantly express a characteristic MSC cell surface phenotype by being positive for CD29, CD44, CD73, CD105, CD90, and HLA-ABC and negative for CD34, CD45, and HLA-DR [110]. Other studies have confirmed these findings, with the exception of a study by Garfias *et al.* [112], who noted marked expression of CD34 despite using cultures between passage numbers 3 and 5. We have demonstrated that subtle changes in limbal stromal cell phenotype occur according to the culture conditions used [111]. While cells in cultures supplemented with serum expressed high levels of CD146 (80%), cells in parallel cultures established using a commercial serum-free culture system (MesenCult-XF®, StemCell Technologies) expressed negligible levels. This disparity in CD146 expression is difficult to explain in view of the acknowledged utility of CD146 as a marker of MSCs that form part of blood vessel walls [115].

A second important observation in the initial study by Polisetty *et al.* was that cultures established from limbal stroma displayed histochemical evidence of differentiation into cells of osteogenic and adipogenic lineages when cultured under appropriate culture conditions [108]. Subsequent histochemical and immunocytochemical studies have provided evidence of chondrogenic differentiation [110–112] and neurogenic differentiation [112] potential. The classic trilineage mesenchymal differentiation potential (osetogenic, lipogenic, and chondrogenic) has been confirmed by RT-qPCR [110].

The apparent multipotency of cultured limbal stromal cells, however, presents a dilemma. While the classic trilineage potential of MSCs derived from bone or bone marrow fits well with intended musculoskeletal applications, most clinical applications for limbal stromal cells would presumably not require, or indeed would not desire, this type of activity. Further studies are therefore required to confirm the degree of true plasticity for limbal stromal cells and to better define the conditions under which nonkeratogenic differentiation occurs.

While multipotency may well be a less desirable activity for cultured limbal stromal cells to share with traditional sources of MSCs, the ability to evade immune detection and suppress inflammation and immunity are clearly desirable traits. To this end, it is encouraging that all studies to date have noted that passaged cultures of limbal stromal cells express low to negligible levels of HLA-DR on their cell surface (Table 36.4). Cultured allogeneic limbal stromal cells are therefore less likely to be rejected compared with conventional donor limbal tissue transplants. In addition, Garfias *et al.* have demonstrated that cultured limbal stromal cells, like MSCs, suppress proliferation of peripheral blood lymphocytes in a dose-dependent manner [112]. Further analysis indicated that this immune suppression activity of limbal stromal cells is mediated through the release of TGF-$\beta$1. We have recently confirmed immunosuppressive properties for human limbal stromal cells cultured in either serum-supplemented or serum-free growth media [113]. We have achieved similar results for cells cultivated from the limbal stroma of rabbit corneas, thus providing the foundation

for a preclinical study in this animal model [112]. Interestingly, the abilities to suppress lymphocyte activation and support epithelial colony formation are displayed both within and across species boundaries.

## 36.8 Conclusions and future directions

The delicate and highly organized structure of the cornea means that even low levels of inflammation caused by disease and trauma can lead to significant loss of vision. Restoration of corneal structure, and thus vision, is therefore acutely tied to the inflammatory status of this tissue. The negative effects of inflammation and antigenic load are especially apparent in the case of traditional and emerging cell- and tissue-based therapies for corneal repair, including donor corneal transplants and cultivated epithelial autografts. It is not surprising, therefore, that MSCs have shown significant potential as agents for improving corneal wound healing and improving retention of corneal allografts in animal models. In particular, the mechanism of MSC action appears highly dependent upon the perioperative timing of their administration. Moreover, significant progress has been made in identifying soluble factors responsible for MSC action, including TSG-6. These results encourage advancing to human trials using MSCs or MSC-derived products, especially with respect to improving the retention of donor corneal allografts.

MSC-based therapy also has significant potential to repair the corneal epithelial stem cell niche by grafting whole segments of donor corneal limbus. However, there remain issues for further exploration, including the optimization of MSC dosage and the route of administration in human patients. The question of whether or not MSCs display keratogenic differentiation is interesting, but we consider this to be less important than progressing to clinical development of this new therapy.

Additionally, the apparent existence of limbal stromal cells with MSC-like properties paves the way for novel therapies based on the isolation and expansion of MSCs from donor limbal tissue that would otherwise be discarded. Further research is required to evaluate the efficacy of these potential limbal MSCs in comparison with *bona fide* MSCs derived from traditional tissue sources such as bone marrow and adipose tissue.

Given that the limbus has a resident stem/stromal cell niche and that several laboratories have already developed different techniques for culturing small explanted biopsies on either biological or synthetic scaffolds prior to implanting them in place of traditional corneal grafts, the ophthalmologic field is already well advanced in the application of novel tissue-engineered approaches that use autologous or allogeneic MSC-differentiated cells *ex vivo*. It will be important to apply good manufacturing practice to the manufacturing of such new treatments with the safety that standard operating procedures and protocols, quality control, and quality assurance provide for the product, the donor, the recipient, the manufacturers of the product, and the institutions sponsoring clinical trials with them.

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## References

1 Coster, D.J. History of corneal transplantation in Australia. *Clin Experiment Ophthalmol* 2015;**43**(3):268–276.
2 Angunawela, R.I., Mehta, J.S., and Daniels, J.T. *Ex-vivo* ocular surface stem cell therapies: current techniques, applications, hurdles and future directions. *Expert Rev Mol Med* 2013;**15**:e4.
3 Harkin, D.G., Apel, A.J., Di Girolamo, N., *et al.* Current status and future prospects for cultured limbal tissue transplants in Australia and New Zealand. *Clin Experiment Ophthalmol* 2013;**41**(3):272–281.
4 Pellegrini, G., Traverso, C.E., Franzi, A.T., *et al.* Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet* 1997;**349**(9057):990–903.
5 Schwab, I.R. Cultured corneal epithelia for ocular surface disease. *Trans Am Ophthalmol Soc* 1999;**97**:891–986.
6 Williams, K.A. and Coster, D.J. The immunobiology of corneal transplantation. *Transplantation* 2007;**84**(7):806–813.
7 Williams, K.A., Esterman, A.J., Bartlett, C., *et al.* How effective is penetrating corneal transplantation? Factors influencing long-term outcome in multivariate analysis. *Transplantation* 2006;**81**(6):896–901.
8 Ozerdem, U., Alitalo, K., Salven, P., and Li, A. Contribution of bone marrow-derived pericyte precursor cells to corneal vasculogenesis. *Invest Ophthalmol Vis Sci* 2005;**46**(10):3502–3506.
9 Wilson, S.E., Mohan, R.R., Netto, M., *et al.* RANK, RANKL, OPG, and M-CSF expression in stromal cells during corneal wound healing. *Invest Ophthalmol Vis Sci* 2004;**45**(7):2201–2211.
10 Lan, Y., Kodati, S., Lee, H.S., *et al.* Kinetics and function of mesenchymal stem cells in corneal injury. *Invest Ophthalmol Vis Sci* 2012;**53**(7):3638–3644.
11 Ye, J., Yao, K., and Kim, J.C. Mesenchymal stem cell transplantation in a rabbit corneal alkali burn model: engraftment and involvement in wound healing. *Eye (Lond)* 2006;**20**(4):482–490.

12 Arnalich-Montiel, F., Pastor, S., Blazquez-Martinez, A., *et al.* Adipose-derived stem cells are a source for cell therapy of the corneal stroma. *Stem Cells* 2008;**26**(2):570–579.

13 Liu, H., Zhang, J., Liu, C.Y., *et al.* Cell therapy of congenital corneal diseases with umbilical mesenchymal stem cells: lumican null mice. *PLoS ONE* 2010;**5**(5):e10707.

14 Monteiro, B.G., Serafim, R.C., Melo, G.B., *et al.* Human immature dental pulp stem cells share key characteristic features with limbal stem cells. *Cell Prolif* 2009;**42**(5):587–594.

15 Schermer, A., Galvin, S., and Sun, T.T. Differentiation-related expression of a major 64K corneal keratin *in vivo* and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol* 1986;**103**(1):49–62.

16 Fini, M.E. Keratocyte and fibroblast phenotypes in the repairing cornea. *Prog Retin Eye Res* 1999;**18**(4):529–551.

17 Joyce, N.C. Proliferative capacity of the corneal endothelium. *Prog Retin Eye Res* 2003;**22**(3):359–389.

18 Smolin, G., Foster, C.S., Azar, D.T., and Dohlman, C.H. (eds). *Smolin and Thoft's The Cornea: Scientific Foundations and Clinical Practice*, 4th edn. Lippincott Williams & Wilkins: Philadelphia, PA, 2005.

19 Agorogiannis, G.I., Alexaki, V.I., Castana, O., and Kymionis, G.D. Topical application of autologous adipose-derived mesenchymal stem cells (MSCs) for persistent sterile corneal epithelial defect. *Graefes Arch Clin Exp Ophthalmol* 2012;**250**(3):455–457.

20 Dua, H.S., Saini, J.S., Azuara-Blanco, A., and Gupta, P. Limbal stem cell deficiency: concept, aetiology, clinical presentation, diagnosis and management. *Indian J Ophthalmol* 2000;**48**(2):83–92.

21 Davidson, A.E., Hayes, S., Hardcastle, A.J., and Tuft, S.J. The pathogenesis of keratoconus. *Eye (Lond)* 2014;**28**(2):189–195.

22 Nanavaty, M.A., Wang, X., and Shortt, A.J. Endothelial keratoplasty versus penetrating keratoplasty for Fuchs endothelial dystrophy. *Cochrane Database Syst Rev*, 2014;**2**:CD008420.

23 Williams, K.A., Muehlberg, S.M., Lewis, R.F., and Coster, D.J. How successful is corneal transplantation? A report from the Australian Corneal Graft Register. *Eye (Lond)* 1995;**9**(Pt 2):219–227.

24 Williams, K.A., Lowe, M.C., Keane, M.C., *et al.* (eds). *The Australian Corneal Graft Registry 2012 Report.* Flinders University: Adelaide.

25 Kenyon, K.R. and Tseng, S.C. Limbal autograft transplantation for ocular surface disorders. *Ophthalmology* 1989;**96**(5):709–722, discussion 722–723.

26 Rheinwald, J.G. and Green, H. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell* 1975;**6**(3):331–343.

27 Tsai, R.J., Li, L.M., and Chen, J.K. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med* 2000;**343**(2):86–93.

28 Du, Y., Carlson, E.C., Funderburgh, M.L., *et al.* Stem cell therapy restores transparency to defective murine corneas. *Stem Cells* 2009;**27**(7):1635–1642.

29 Hamrah, P., Zhang, Q., Liu, Y., and Dana, M.R. Novel characterization of MHC class II-negative population of resident corneal Langerhans cell-type dendritic cells. *Invest Ophthalmol Vis Sci* 2002;**43**(3):639–646.

30 Hamrah, P. and Dana, M.R. Corneal antigen-presenting cells. *Chem Immunol Allergy* 2007;**92**:58–70.

31 Hamrah, P., Huq, S.O., Liu, Y., *et al.* Corneal immunity is mediated by heterogeneous population of antigen-presenting cells. *J Leukoc Biol* 2003;**74**(2):172–178.

32 Niederkorn, J.Y. Immune privilege in the anterior chamber of the eye. *Crit Rev Immunol* 2002;**22**(1):13–46.

33 Williams, K.A., Ash, J.K., and Coster, D.J. Histocompatibility antigen and passenger cell content of normal and diseased human cornea. *Transplantation* 1985;**39**(3):265–269.

34 Williams, K.A., White, M.A., Ash, J.K., and Coster, D.J. Leukocytes in the graft bed associated with corneal graft failure. Analysis by immunohistology and actuarial graft survival. *Ophthalmology* 1989;**96**(1):38–44.

35 Coster, D.J. and Williams, K.A. The impact of corneal allograft rejection on the long-term outcome of corneal transplantation. *Am J Ophthalmol* 2005;**140**(6):1112–1122.

36 Frost, N.A., Wu, J., Lai, T.F., and Coster, D.J. A review of randomized controlled trials of penetrating keratoplasty techniques. *Ophthalmology* 2006;**113**(6):942–949.

37 McMenamin, P.G., Crewe, J., Morrison, S., and Holt, P.G. Immunomorphologic studies of macrophages and MHC class II-positive dendritic cells in the iris and ciliary body of the rat, mouse, and human eye. *Invest Ophthalmol Vis Sci* 1994;**35**(8):3234–3250.

38 Steptoe, R.J., McMenamin, P.G., and Holt, P.G. Resident tissue macrophages within the normal rat iris lack immunosuppressive activity and are effective antigen-presenting cells. *Ocul Immunol Inflamm* 2000;**8**(3):177–187.

39 Catry, L., Van den Oord, J., Foets, B., and Missotten, L. Morphologic and immunophenotypic heterogeneity of corneal dendritic cells. *Graefes Arch Clin Exp Ophthalmol* 1991;**229**(2):182–185.

40 Bertelmann, E., Jaroszewski, J., and Pleyer, U. Corneal allograft rejection: current understanding. 2. Clinical implications. *Ophthalmologica* 2002;**216**(1):2–12.

41 Liu, Y., Hamrah, P., Zhang, Q., *et al.* Draining lymph nodes of corneal transplant hosts exhibit evidence for donor major histocompatibility complex (MHC) class II-positive dendritic cells derived from MHC class II-negative grafts. *J Exp Med* 2002;**195**(2):259–268.

42 Cursiefen, C., Schlötzer-Schrehardt, U., Küchle, M., *et al.* Lymphatic vessels in vascularized human corneas: immunohistochemical investigation using LYVE-1 and podoplanin. *Invest Ophthalmol Vis Sci* 2002;**43**(7):2127–2135.

43 Maruyama, K., Ii, M., Cursiefen, C., *et al.* Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* 2005;**115**(9):2363–2372.

44 Xu, H., Chen, M., Reid, D.M., and Forrester, J.V. LYVE-1-positive macrophages are present in normal murine eyes. *Invest Ophthalmol Vis Sci* 2007;**48**(5):2162–2171.

45 Claerhout, I., Kestelyn, P., Debacker, V., *et al.* Role of natural killer cells in the rejection process of corneal allografts in rats. *Transplantation* 2004;**77**(5):676–682.

46 Hegde, S. and Niederkorn, J.Y. The role of cytotoxic T lymphocytes in corneal allograft rejection. *Invest Ophthalmol Vis Sci* 2000;**41**(11):3341–3347.

47 Larkin, D.F., Alexander, R.A., and Cree, I.A. Infiltrating inflammatory cell phenotypes and apoptosis in rejected human corneal allografts. *Eye (Lond)* 1997;**11**(Pt 1):68–74.

48 Williams, K.A., Standfield, S.D., Mills, R.A., *et al.* A new model of orthotopic penetrating corneal transplantation in the sheep: graft survival, phenotypes of graft-infiltrating cells and local cytokine production. *Aust N Z J Ophthalmol* 1999;**27**(2):127–135.

49 Conget, P.A. and Minguell, J.J. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 1999;**181**(1):67–73.

50 Izadpanah, R., Trygg, C., Patel, B., *et al*. Biologic properties of mesenchymal stem cells derived from bone marrow and adipose tissue. *J Cell Biochem* 2006;**99**(5):1285–1297.

51 Di Nicola, M., Carlo-Stella, C., Magni, M., *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**(10): 3838–3843.

52 Glennie, S., Soeiro, I., Dyson, P.J., *et al*. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005;**105**(7):2821–2827.

53 Le Blanc, K. Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 2003;**5**(6):485–489.

54 Aggarwal, S. and Pittenger, M.F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4): 1815–1822.

55 Zhang, W., Ge, W., Li, C., *et al*. Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. *Stem Cells Dev* 2004;**13**(3):263–271.

56 Nauta, A.J., Kruisselbrink, A.B., Lurvink, E., *et al*. Mesenchymal stem cells inhibit generation and function of both $CD34^+$-derived and monocyte-derived dendritic cells. *J Immunol* 2006;**177**(4): 2080–2087.

57 Klyushnenkova, E., Mosca, J.D., Zernetkina, V., *et al*. T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* 2005;**12**(1): 47–57.

58 Rasmusson, I., Ringdén, O., Sundberg, B., and Le Blanc, K. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 2003;**76**(8):1208–1213.

59 Corcione, A., Benvenuto, F., Ferretti, E., *et al*. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**(1):367–372.

60 Asari, S., Itakura, S., Ferreri, K., *et al*., esenchymal stem cells suppress B-cell terminal differentiation. *Exp Hematol* 2009;**37** (5):604–615.

61 Krampera, M., Cosmi, L., Angeli, R., *et al*. Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**(2):386–398.

62 Le Blanc, K., Frassoni, F., Ball, L., *et al*. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**(9624):1579–1586.

63 Van Poll, D., Parekkadan, B., Cho, C.H., *et al*., Mesenchymal stem cell-derived molecules directly modulate hepatocellular death and regeneration in vitro and *in vivo*. *Hepatology* 2008;**47**(5):1634–1643.

64 Gonzalez-Rey, E., Gonzalez, M.A., Varela, N., *et al*. Human adipose-derived mesenchymal stem cells reduce inflammatory and T cell responses and induce regulatory T cells *in vitro* in rheumatoid arthritis. *Ann Rheum Dis* 2010;**69**(1):241–248.

65 Németh, K., Leelahavanichkul, A., Yuen, P.S., *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**(1):42–49.

66 Dazzi, F., Lopes, L., and Weng, L. Mesenchymal stromal cells: a key player in "innate tolerance"? *Immunology* 2012;**137**(3):206–213.

67 Omoto, M., Miyashita, H., Shimmura, S., *et al*. The use of human mesenchymal stem cell-derived feeder cells for the cultivation of transplantable epithelial sheets. *Invest Ophthalmol Vis Sci* 2009; **50**(5):2109–2115.

68 Nakahara, M., Okumura, N., Kay, E.P., *et al*. Corneal endothelial expansion promoted by human bone marrow mesenchymal stem cell-derived conditioned medium. *PLoS ONE* 2013;**8**(7): e69009.

69 Oh, J.Y., Kim, M.K., Shin, M.S., *et al*. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* 2008;**26**(4): 1047–1055.

70 Zajicova, A., Pokorna, K., Lencova, A., *et al*. Treatment of ocular surface injuries by limbal and mesenchymal stem cells growing on nanofiber scaffolds. *Cell Transplant* 2010;**19**(10):1281–1290.

71 Gomes, J.A., Geraldes Monteiro, B., Melo, G.B., *et al*. Corneal reconstruction with tissue-engineered cell sheets composed of human immature dental pulp stem cells. *Invest Ophthalmol Vis Sci* 2010;**51**(3):1408–1414.

72 Roddy, G.W., Oh, J.Y., Lee, R.H., *et al*. Action at a distance: systemically administered adult stem/progenitor cells (MSCs) reduce inflammatory damage to the cornea without engraftment and primarily by secretion of TNF-α stimulated gene/protein 6. *Stem Cells* 2011;**29**(10):1572–1579.

73 Yao, L., Li, Z.R., Su, W.R., *et al*. Role of mesenchymal stem cells on cornea wound healing induced by acute alkali burn. *PLoS ONE* 2012;**7**(2):e30842.

74 Cejkova, J., Trosan, P., Cejka, C., *et al*. Suppression of alkali-induced oxidative injury in the cornea by mesenchymal stem cells growing on nanofiber scaffolds and transferred onto the damaged corneal surface. *Exp Eye Res* 2013;**116**:312–323.

75 Lin, H.F., Lai, Y.C., Tai, C.F., *et al*. Effects of cultured human adipose-derived stem cells transplantation on rabbit cornea regeneration after alkaline chemical burn. *Kaohsiung J Med Sci* 2013; **29**(1):14–18.

76 Zeppieri, M., Salvetat ML, Beltrami AP, *et al*. Human adipose-derived stem cells for the treatment of chemically burned rat cornea: preliminary results. *Curr Eye Res* 2013;**38**(4):451–463.

77 Pinarli, F.A., Okten, G., Beden, U., *et al*. Keratinocyte growth factor-2 and autologous serum potentiate the regenerative effect of mesenchymal stem cells in cornea damage in rats. *Int J Ophthalmol* 2014;**7**(2):211–219.

78 Javorkova, E., Trosan, P., Zajicova, A., *et al*. Modulation of the early inflammatory microenvironment in the alkali-burned eye by systemically administered interferon-γ-treated mesenchymal stromal cells. *Stem Cells Dev* 2014;**23**(20):2490–2500.

79 Lee, J.Y., Jeong, H.J., Kim, M.K., and Wee, W.R. Bone marrow-derived mesenchymal stem cells affect immunologic profiling of interleukin-17-secreting cells in a chemical burn mouse model. *Korean J Ophthalmol* 2014;**28**(3):246–256.

80 Oh, J.Y., Kim, M.K., Ko, J.H., *et al*. Rat allogeneic mesenchymal stem cells did not prolong the survival of corneal xenograft in a pig-to-rat model. *Vet Ophthalmol* 2009;**12**(Suppl 1):35–40.

81 Jia, Z., Jiao, C., Zhao, S., *et al*. Immunomodulatory effects of mesenchymal stem cells in a rat corneal allograft rejection model. *Exp Eye Res* 2012;**102**:44–49.

82 Oh, J.Y., Lee, R.H., Yu, J.M., *et al*. Intravenous mesenchymal stem cells prevented rejection of allogeneic corneal transplants by aborting the early inflammatory response. *Mol Ther* 2012;**20** (11):2143–2152.

83 Lee, H.J., Ko, J.H., Ko, A.Y., *et al*. Intravenous infusion of mesenchymal stem/stromal cells decreased $CCR7^+$ antigen presenting

cells in mice with corneal allotransplantation. *Curr Eye Res* 2014; **39**(8):780–789.

84 Omoto, M., Katikireddy, K.R., Rezazadeh, A., *et al*. Mesenchymal stem cells home to inflamed ocular surface and suppress allosensitization in corneal transplantation. *Invest Ophthalmol Vis Sci* 2014;**55**(10):6631–6638.

85 Treacy, O., O'Flynn, L., Ryan, A.E., *et al*. Mesenchymal stem cell therapy promotes corneal allograft survival in rats by local and systemic immunomodulation. *Am J Transplant* 2014;**14**(9): 2023–2036.

86 Ma, Y., Xu, Y., Xiao, Z., *et al*. Reconstruction of chemically burned rat corneal surface by bone marrow-derived human mesenchymal stem cells. *Stem Cells* 2006;**24**(2):315–321.

87 Guo, T., Wang, W., Zhang, J., *et al*. Experimental study on repairing damage of corneal surface by mesenchymal stem cells transplantation. *Zhonghua Yan Ke Za Zhi* 2006;**42**(3):246–250 (in Chinese).

88 Gu, S., Xing, C., Han, J., *et al*. Differentiation of rabbit bone marrow mesenchymal stem cells into corneal epithelial cells *in vivo* and *ex vivo*. *Mol Vis* 2009;**15**:99–107.

89 Zhang, J., Huang, C., Feng, Y., *et al*. Comparison of beneficial factors for corneal wound-healing of rat mesenchymal stem cells and corneal limbal stem cells on the xenogeneic acellular corneal matrix *in vitro*. *Mol Vis* 2012;**18**:161–173.

90 Rohaina, C.M., Then, K.Y., Ng, A.M., *et al*. Reconstruction of limbal stem cell deficient corneal surface with induced human bone marrow mesenchymal stem cells on amniotic membrane. *Transl Res* 2014;**163**(3):200–210.

91 Katikireddy, K.R., Dana, R., and Jurkunas, U.V. Differentiation potential of limbal fibroblasts and bone marrow mesenchymal stem cells to corneal epithelial cells. *Stem Cells* 2014;**32**(3):717–729.

92 Du, Y., Roh, D.S., Funderburgh, M.L., *et al*. Adipose-derived stem cells differentiate to keratocytes *in vitro*. *Mol Vis* 2010;**16**:2680–2689.

93 Liu, H., Zhang, J., Liu, C.Y., *et al*. Bone marrow mesenchymal stem cells can differentiate and assume corneal keratocyte phenotype. *J Cell Mol Med* 2012;**16**(5):1114–1124.

94 Liu, X.W. and Zhao, J.L. Transplantation of autologous bone marrow mesenchymal stem cells for the treatment of corneal endothelium damages in rabbits. *Zhonghua Yan Ke Za Zhi* 2007;**43**(6):540–545 (in Chinese).

95 Joyce, N.C., Harris, D.L., Markov, V., *et al*. Potential of human umbilical cord blood mesenchymal stem cells to heal damaged corneal endothelium. *Mol Vis* 2012;**18**:547–564.

96 Dave, S.D., Vanikar, A.V., and Trivedi, H.L. *In-vitro* generation of human adipose tissue derived insulin secreting cells: up-regulation of Pax-6, Ipf-1 and Isl-1. *Cytotechnology* 2014;**66**(2):299–307.

97 Garzón, I., Martín-Piedra, M.A., Alfonso-Rodríguez, C., *et al*. Generation of a biomimetic human artificial cornea model using Wharton's jelly mesenchymal stem cells. *Invest Ophthalmol Vis Sci* 2014;**55**(7):4073–4083.

98 Jiang, T.S., Cai, L., Ji, W.Y., *et al*. Reconstruction of the corneal epithelium with induced marrow mesenchymal stem cells in rats. *Mol Vis* 2010;**16**:1304–1316.

99 Reinshagen, H., Auw-Haedrich, C., Sorg, R.V., *et al*. Corneal surface reconstruction using adult mesenchymal stem cells in experimental limbal stem cell deficiency in rabbits. *Acta Ophthalmol* 2011; **89**(8):741–748.

100 Coulson-Thomas, V.J., Caterson, B., and Kao, W.W. Transplantation of human umbilical mesenchymal stem cells cures the corneal defects of mucopolysaccharidosis VII mice. *Stem Cells* 2013; **31**(10):2116–2126.

101 Espandar, L., Bunnell, B., Wang, G.Y., *et al.*, dipose-derived stem cells on hyaluronic acid-derived scaffold: a new horizon in bioengineered cornea. *Arch Ophthalmol* 2012;**130**(2):202–208.

102 Ma, X.Y., Bao, H.J., Cui, L., and Zou, J. The graft of autologous adipose-derived stem cells in the corneal stromal after mechanic damage. *PLoS ONE* 2013;**8**(10):e76103.

103 Ainscough, S.L. Improved strategies for the cultivation of human limbal epithelial (HLE) grafts. PhD thesis, School of Life Sciences, Queensland University of Technology: Brisbane, 2008.

104 Choong, P.F., Mok, P.L., Cheong, S.K., and Then, K.Y. Mesenchymal stromal cell-like characteristics of corneal keratocytes. *Cytotherapy* 2007;**9**(3):252–258.

105 Du, Y., Funderburgh, M.L., Mann, *et al*. Multipotent stem cells in human corneal stroma. *Stem Cells* 2005;**23**(9):1266–1275.

106 Shimmura, S. and Kawakita, T. Accessory cell populations in the cornea. *Ocul Surf* 2006;**4**(2):74–80.

107 Ainscough, S.L., Linn, M.L., Barnard, Z., *et al*. Effects of fibroblast origin and phenotype on the proliferative potential of limbal epithelial progenitor cells. *Exp Eye Res* 2011;**92**(1):10–19.

108 Polisetty, N., Fatima, A., Madhira, S.L., *et al*. Mesenchymal cells from limbal stroma of human eye. *Mol Vis* 2008;**14**:431–442.

109 Polisetti, N., Agarwal, P., Khan, I., *et al*. Gene expression profile of epithelial cells and mesenchymal cells derived from limbal explant culture. *Mol Vis* 2010;**16**:1227–1240.

110 Branch, M.J., Hashmani, K., Dhillon, P., *et al*. Mesenchymal stem cells in the human corneal limbal stroma. *Invest Ophthalmol Vis Sci* 2012;**53**(9):5109–5116.

111 Bray, L.J., Heazlewood, C.F., Atkinson, K., *et al*. Evaluation of methods for cultivating limbal mesenchymal stromal cells. *Cytotherapy* 2012;**14**(8):936–947.

112 Garfias, Y., Nieves-Hernandez, J., Garcia-Mejia, M., *et al*. Stem cells isolated from the human stromal limbus possess immunosuppressant properties. *Mol Vis* 2012;**18**:2087–2095.

113 Bray, L.J., Heazlewood, C.F., Munster, D.J., *et al*. Immunosuppressive properties of mesenchymal stromal cell cultures derived from the limbus of human and rabbit corneas. *Cytotherapy* 2014;**16**(1):64–73.

114 Basu, S., Hertsenberg, A.J., Funderburgh, M.L, *et al.*, Human limbal biopsy-derived stromal stem cells prevent corneal scarring. *Sci Transl Med* 2014;**6**(266):266ra172.

115 Crisan, M., Yap, S., Casteilla, L., *et al*. A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.

CHAPTER 37

# The role of paracrine factors secreted by mesenchymal stromal cells in acute tissue injury

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## 37.1 Introduction

True mesenchymal *stem* cells are defined by their self-renewal ability and their differentiation potential into mesodermal cell types. They are, however, rare cells with a frequency in bone marrow of between 1 in $10^4$ and 1 in $10^5$. This makes it difficult to obtain sufficient numbers of them for most experimental purposes, and certainly for clinical trials. Therefore, their progeny, undifferentiated mesenchymal *stromal* cells (MSCs), which can be readily expanded in large numbers, have been used for these purposes. MSCs form part of the endothelial wall of blood vessels and thus can be isolated from any vascularized organ or tissue.

MSCs are believed to hold great promise in cell replacement therapy. Recent studies, however, have shown that cell empowerment, rather than replacement, is more critical. This notion has increasingly attracted attention and is thought to be a key for understanding MSC-based therapy other than that aimed at regenerating musculoskeletal tissues, in which bone and tendon transdifferentiation has been shown to be the mechanism of action. It is envisioned that concerted actions of MSCs on immune responses and tissue repair are exerted through paracrine factors, including immunoregulatory factors, cell mobility factors, and growth factors. In this chapter we discuss the key roles of these paracrine factors in tissue repair and regeneration.

## 37.2 Cell replacement and cell empowerment

Preclinical studies have suggested that MSCs are effective in a number of acute tissue injuries, including myocardial infarction [1–4], sepsis [5,6], hepatic failure [7], lung injuries [8–15], peritonitis [16] and acute experimental autoimmune encephalomyelitis (EAE) [17]. In experiments tracing *ex vivo* expanded MSCs infused intravenously, it was shown that MSCs became trapped in the lungs initially, but were subsequently recruited to damaged tissues such as infarcted myocardium, traumatic brain injury, fibrotic liver, damaged lung, and tumors [18].

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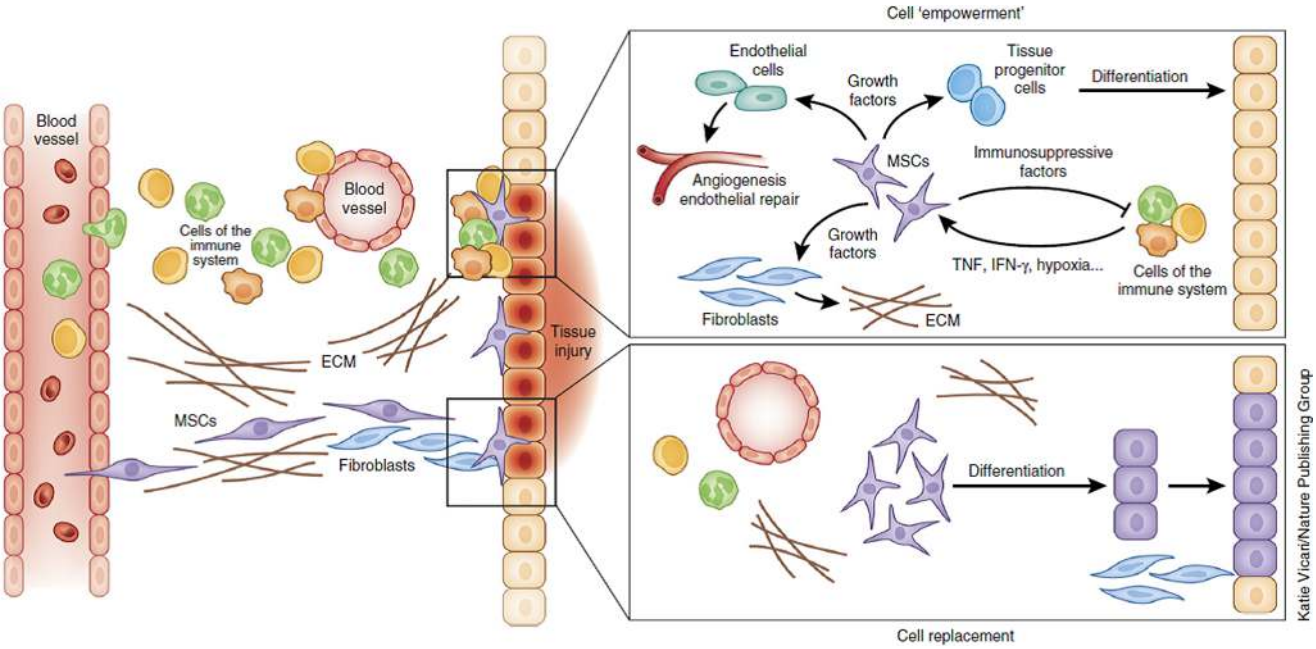



**Figure 37.1** Modes of MSC-based therapy: cell replacement versus cell "empowerment." When tissue is damaged, inflammation occurs and MSCs are mobilized to the site. Since they have multipotent differentiation potential, the recruited MSCs are believed to differentiate into functional cells to replace damaged cells. However, in response to inflammatory cytokines, MSCs also help prepare the microenvironment by producing immunoregulatory factors that modulate the progression of inflammation. MSCs also produce large amounts of growth factors that subsequently stimulate endothelial cells, fibroblasts, and, most importantly, tissue progenitor cells *in situ*. The concerted action of these factors and cells facilitates tissue repair through angiogenesis, remodeling of the extracellular matrix (ECM), and the differentiation of tissue progenitor cells. (See color plate section.) *Source*: Wang *et al. Nature Immunology* 2014; 15:1009–1016. Reproduced with permission.

Importantly, endogenous nestin$^+$ MSCs have been observed to migrate from bone marrow to asthmatic lungs [19]. After homing to injured tissues, MSC-based therapy can be achieved in two ways: cell replacement and cell "empowerment" (Figure 37.1) [20].

Based on their proliferative and mesodermal differentiation capability, MSCs are being used for their potential to replace damaged cells such as cardiomyocytes, hepatocytes, and endothelial cells, thereby promoting tissue regeneration in various organs, such as the heart, lung, and liver [21–24]. Growing evidence from both clinical and preclinical studies in arthritis, liver cirrhosis, myocardial infarction, renal degeneration, and neural degeneration have demonstrated that the beneficial role of MSCs in disease treatment is not related to the efficiency of cell engraftment and the longevity of MSCs [4,25–29]. Rather, factors such as hepatic growth factor (HGF) and tumor-necrosis-factor-(TNF)-inducible gene 6 (TSG-6) protein, which form part of the MSC secretome, or MSC conditioned medium alone, may substitute for the presence of MSCs themselves and exert beneficial effects on various inflammatory diseases [4,29,30]. Furthermore, in preclinical models, autologous, allogeneic, and xenogeneic MSCs are effective in treating tissue injuries, even in immunocompetent recipients, suggesting that MSC engraftment is not necessary for the mediation of therapeutic benefit in many circumstances [25,31]. Thus, the cell empowerment model is more important than the cell replacement model for MSC-based therapy, at least when diseases other than those of the musculoskeletal system are involved.

Through concerted actions of their secreted immunosuppressive factors, cytokines, growth factors, and differentiation factors, MSCs suppress inflammation, promote tissue progenitor cell proliferation and differentiation, facilitate endothelial cell function and fibroblast activity, and maintain tissue homeostasis [20]. Various studies have demonstrated that the production of paracrine factors is dependent on inflammatory responses.

## 37.3 Paracrine factors produced by mesenchymal stromal cells

Damaged tissue is always accompanied by infiltration of cells of the innate immune system, including macrophages and neutrophils, as well as those of the adaptive immune system, including T cells and B cells (see Chapters 33 and 34). These cells are recruited by chemotactic factors released from apoptotic cells, necrotic cells, damaged microvasculature and stroma [32,33]. Accumulation of these immune cells subsequently induces the production of inflammatory mediators, such as TNF-α, interleukin (IL)-1β, interferon (IFN)-γ, IL-17, free radicals, and chemokines, depending on the nature of the inflammatory stimulus [34]. These inflammatory molecules and immune system cells,

together with endothelial cells and fibroblasts, constitute the tissue microenvironment that influences the properties of MSCs during tissue repair [35]. The mechanisms by which MSCs are recruited, survive, and differentiate in damaged tissues have not been well understood until recently. However, investigations have found that MSCs stimulated by inflammatory signals such as cytokines, toxins of infectious agents, hypoxia, and toll-like receptor (TLR) ligands can secrete an array of growth factors and distinct soluble immunosuppressive factors [35,36]. We here summarize the effects of these paracrine factors on tissue repair processes.

### 37.3.1 Growth factors and mesenchymal-stromal-cell-mediated tissue repair

During acute tissue damage the endothelial cell barrier is broken down, allowing infiltration of leukocytes and the release of protein-rich plasma from blood into the damaged area [35]. The supporting function of fibroblasts becomes abnormal, resulting in aberrant extracellular matrix deposition and tissue integrity. Some studies have indicated that growth factors produced by MSCs are important in treating wounds and in improving recovery from myocardial infarction [37–39]. There is accumulating evidence that MSCs stimulated by inflammatory signals can secrete many growth factors, including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor, transforming growth factor-β (TGF-β), vascular endothelial growth factor, HGF, insulin growth factor-1, angiopoietin-1 (Ang-1), keratinocyte growth factor, and stromal cell-derived factor-1 (also known as C–X–C motif chemokine ligand 12) [36,40–42]. These factors have been linked to the tissue repair functions of MSCs by promoting the activation and differentiation of tissue progenitor cells and the development of endothelial cells and fibroblasts. Among these growth factors, Ang-1, vascular endothelial growth factor, HGF, EGF, platelet-derived growth factor, FGF, keratinocyte growth factor, and TGF-β can directly regulate endothelial cells. Through promoting endothelial cell proliferation, regulating ECM production, decreasing endothelial permeability, and inhibiting the interaction between leukocytes and endothelial cells, these factors can maintain endothelial integrity and promote angiogenesis [43,44]. Additionally, they can influence fibroblasts to maintain tissue integrity and promote wound healing.

In addition to their functions on endothelial cells and fibroblasts, growth factors secreted by MSCs have also been demonstrated to have a role in the restoration of intrinsic tissue progenitor cells. For instance, it has been reported that HGF secreted by MSCs is effective in modulating endogenous neural cell remyelination, enabling recovery from EAE and spinal cord demyelination [29]. Other factors, such as FGF, stem cell factor, macrophage colony-stimulating factor, stromal cell-derived factor-1, leukemia inhibitory factor, Ang-1, and chemokines can also stimulate intrinsic tissue repair. Taken together, these observations demonstrate the complicated interactions between MSCs and damaged tissue during the process of tissue repair. The multitude of paracrine factors produced by MSCs, which provoke tissue resident progenitor cells or other relevant cells to initiate tissue repair, are in part responsible for the therapeutic effects of MSCs on tissue repair, even in the absence of local MSC engraftment [29,35,45].

### 37.3.2 Soluble immunosuppressive factors and mesenchymal-stromal-cell-mediated tissue repair

Cellular components released from necrotic cells in injured tissue are first sensed by resident immune cells such as macrophages, which are activated and subsequently produce various inflammatory molecules to initiate and promote local inflammation. Additional innate immune system cells, such as neutrophils, and adaptive immune system cells, such as B cells, $CD4^+$ T cells, and $CD8^+$ T cells, are also attracted to the site of tissue injury and augment the inflammation. Thus, downmodulating an exuberant and excessive inflammatory infiltrate, such as the massive infiltration by neutrophils and macrophages that occurs in infarcted myocardium, can prevent the subsequent replacement of myocardial muscle by fibroblasts and help avoid the consequent hypokinesis of the left ventricular wall that unchecked will lead to refractory heart failure.

One of the properties of MSC-based tissue repair is their immunosuppressive ability. MSCs have been shown to inhibit several types of immune cells, including T cells, B cells, dendritic cells, and natural killer (NK) cells at several key steps [36,46–48] (Figure 37.2).

Since the discovery that bone-marrow-derived MSCs could suppress T cell proliferation in 1998, the immunosuppressive ability of those cells on T cells has been actively investigated [48–50]. The inhibitory effects of MSCs on general T cell activation have been reported in both *in vitro* and *in vivo* experimental systems. Recent studies showed that MSCs can specifically inhibit T cell differentiation into Th1 and Th17 subsets of helper T cells and enhance the generation of Tregs [51]. Their regulation of Tregs involves induction of dendritic cells to a tolerogenic phenotype, which subsequently elicits the generation of Tregs [52]. Moreover, the induction of Tregs by MSCs can result from high levels of TGF-β production by macrophages that are activated by apoptotic T cells [53]. The switching from a proinflammatory to an anti-inflammatory status can also be observed in macrophages. In MSC-based sepsis treatment, MSCs can promote proinflammatory macrophages to switch into anti-inflammatory macrophages [5,54]. Also, MSCs can retain dendritic cells in an immature state via suppressing the expression of MHC class II, CD1α, CD40, CD80, and CD86, and decreasing proinflammatory cytokine production [55,56]. NK cells, one type of innate immune cell, can also be suppressed by MSCs through soluble factors, such as TGF-β and $PGE_2$ [57]. In the human system, similar suppression of IL-2- or IL-15-driven NK cells by MSCs has been observed [58]. Other suppression such as that mediated by MSCs on B cell proliferation are also found [59]. Taken together, MSCs possess extensive immunosuppression

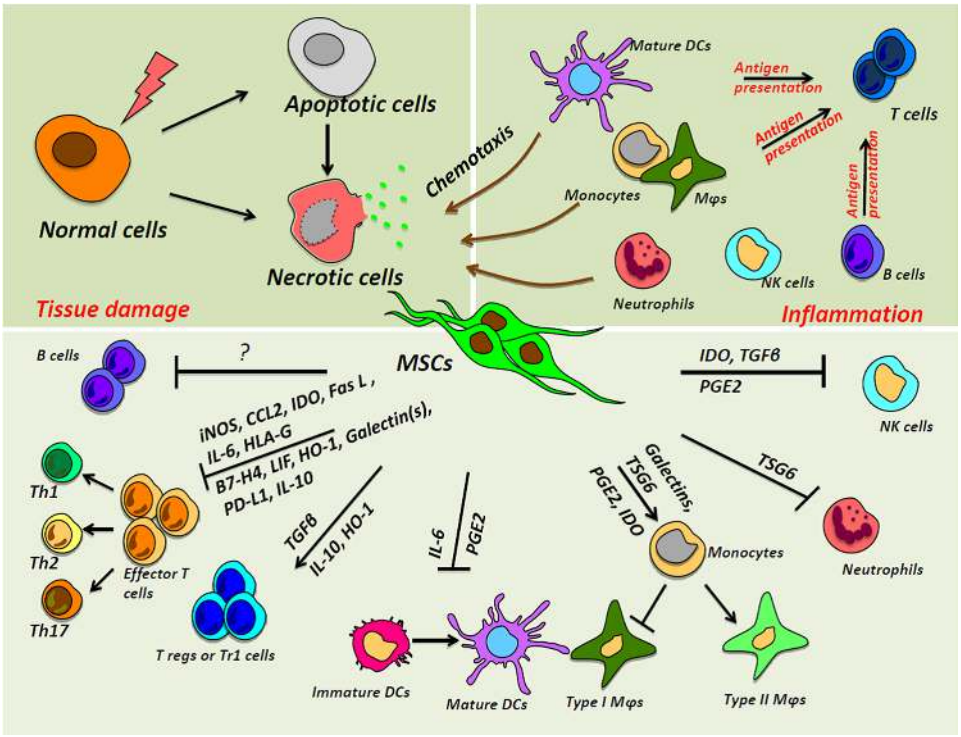



**Figure 37.2** Immunoregulation by MSCs. Immune cells accumulate in damaged tissues and release signals to recruit MSCs. At damaged tissue sites, MSCs have been reported to exert immunosuppressive effects on distinct types of immune cells by expression of immunosuppressive molecules. MSCs inhibit T cell activation and Th1/Th2/Th17 cell differentiation, induce regulatory T cells, render dendritic cells tolerogenic, and induce anti-inflammatory macrophages as well as suppress the activity of B cells, neutrophils, and NK cells. The mechanisms by which MSCs block inflammatory cell activity are shown by the T-shaped symbols. The mechanisms by which MSCs promote effective tissue repair are shown by the arrows. CCL2: chemokine (C–C motif) ligand 2; HLA-G: human leukocyte antigen G; HO-1: heme oxygenase-1; IDO: indoleamine 2,3-dioxygenased; iNOS: inducible nitric oxide synthase; LIF: leukemia inhibitory factor; PD-L1: programmed death ligand 1; PGE2: prostaglandin $E_2$; Th: helper T cell; Tregs: regulatory T cells. (See color plate section.)

ability on immune cells and so have attracted great interest from basic and clinical researchers. Recent investigations have suggested that the immunosuppressive function of MSCs can be enacted through a paracrine effect in a cell–cell contact manner.

MSCs are benign and cannot inhibit immune responses unless they are first activated by certain combinations of inflammatory cytokines. In the presence of IFN-γ, combined with one (or more) other cytokine(s), including TNF-α, IL-1α, or IL-1β, MSCs are licensed to exert their immunosuppressive function [60]. The vital role of IFN-γ and it receptor IFN-γR has been investigated in experiments with antibodies to IFN-γ or to IFN-γR, as well as IFN-γR1-deficient MSCs [60,61]. Under appropriate inflammatory stimulation, MSCs express large amounts of immunoregulatory factors, such as IDO and iNOS, as well as chemokines, including ligands for chemokine receptors CXCR3 and CCR5, which are critical for T cell chemotaxis [60,62]. Through the concerted action of chemokines and immunoregulatory factors, MSCs exert suppressive effects on these recruited T cells. Investigations on the mechanisms of MSC-mediated immunosuppression have demonstrated that not only are cell–cell contact molecules, including FasL and PD-L1/2, important for the functions of these cells, but soluble factors such as nitric oxide (NO), IDO metabolites, and immunosuppressive cytokines are critical for their immunosuppressive effects [35]. These factors are detailed in the following subsections.

### 37.3.3 Inducible nitric oxide synthase/ indoleamine 2,3-dioxygenase

NO in high concentrations has been shown to inhibit immune responses through yet largely undefined mechanisms. iNOS is expressed in murine MSCs when stimulated with the proinflammatory cytokine IFN-γ in combination with either TNF-α or IL-1 [60]. The high concentration of NO suppresses signal transducer and activator of transcription (STAT)5 phosphorylation in T cells and induces immune cell apoptosis [63]. Suppression of iNOS activity by either chemical inhibitors or genetic ablation in MSCs reverses the immunosuppression mediated by MSCs and abolishes the therapeutic effect of MSCs in several immune abnormality models [64]. However, NO is a highly labile oxidative species and can only act on target cells that are in close proximity to cells producing it. Experiments using permeable

membrane to separate lymphocytes and MSCs have demonstrated that the immunosuppressive factor works over a short distance. Indeed, MSC-mediated immunosuppression on T cells through NO is dependent on high chemokine production, which recruits T cells to the vicinity of MSCs and facilitates the suppression by NO on T cells.

Importantly, analysis of immunosuppressive factors between different species shows that MSCs derived from rodents and rabbit use iNOS to exert their immunosuppressive effect, while MSCs derived from human, monkey, and pig employ IDO instead [61,62,65]. IDO is an enzyme that catalyzes tryptophan into kynurenine. The depletion of tryptophan and accumulation of metabolites account for the immunosuppression mediated by IDO [66]. However, the mechanism of IDO-mediated immunosuppression remains unclear. Studies have revealed that IDO is involved in suppressing T cell proliferation, promoting type 2 macrophage differentiation, and impairing NK cell activity [62,67,68].

iNOS and IDO are key factors in mediating the immunoregulation of MSCs. Promoting their expression in MSCs by inflammatory cytokines can license or optimize MSC-mediated immunosuppression, while inhibiting or downregulating their expression in MSCs will enhance the immune response. Thus, strategies targeting iNOS or IDO can modulate the plasticity of MSCs in immune disorders, thereby facilitating MSC-based treatment via decreasing or increasing immune responses [20].

### 37.3.4 Prostaglandin $E_2$

$PGE_2$ is another molecule involved in the immunosuppressive role of MSCs that can be induced by inflammatory cytokines. It has been reported that IL-6-induced $PGE_2$ plays a major role in the therapeutic effect of MSCs in an experimental model of arthritis [69]. Also, $PGE_2$ produced by MSCs is critical for the attenuation of sepsis, which can promote macrophages to produce high levels of IL-10 [5]. Several studies demonstrated that $PGE_2$ secreted by mouse or human MSCs can inhibit dendritic cell maturation, reprogram macrophages to secrete IL-10, shift the balance between Th1 and Th2 T cells, induce Treg cells, and suppress NK cell function [5,57,68,70–72]. Of note, $PGE_2$ can promote IDO-mediated MSC immunosuppression of T cells and NK cells [68,73]. Further studies on the mechanism of $PGE_2$ expression in MSCs and its role in immunosuppression will help facilitate clinical application of MSCs.

### 37.3.5 Tumor-necrosis-factor-inducible gene 6 protein

TSG-6 is an anti-inflammatory protein that is inducible by a subset of inflammatory cytokines, such as TNF-α and IL-1. In patients with inflammatory or autoimmune disorders, TSG-6 has been found to be highly expressed in a variety of cells, suggesting that TSG-6 is involved in the pathogenesis of autoimmune diseases. In MSC-based treatment of myocardial infarction, intravenously injected MSCs accumulate in the lung and form microemboli, which can induce the production of TSG-6 by MSCs. TSG-6 is important in MSC-mediated suppression of the inflammatory response, reduction of infarct size, and improvement of cardiac function [4]. Similarly, immunosuppression mediated by TSG-6 has also been observed in MSC-based treatment of zymosan-induced mouse peritonitis, in which TSG-6 alleviates inflammation through dampening TLR 2/NF-κB signaling in resident macrophages [16]. However, the exact role of TSG-6 in human MSC-mediated immunoregulation requires detailed further investigations.

### 37.3.6 Chemokine (C–C motif) ligand 2

CCL2 is a low molecular weight chemokine. Classically, it is regarded as a molecule that mediates chemotaxis of myeloid cells to inflammatory sites by ligating its receptor, CCR2. An *in vitro* investigation showed that matrix metalloprotease (MMP)-cleaved CCL2 can dramatically decrease monocyte migration [74]. In an experiment using MSCs to treat EAE, it was shown that MSCs could inhibit disease progression via a paracrine conversion of CCL2 from agonist to antagonist to suppress Th17 cells [17]. Similarly, cleaved CCL2 processed by MMP has been demonstrated to mediate the suppression of MSCs on immunoglobulin production by plasma cells. In this process, cleaved CCL2 suppresses the activation of STAT3 and induces the activity of paired box protein 5 in plasma cells [75]. Additionally, intact CCL2 is also important in MSC-mediated tissue repair, especially in tissue injuries that need monocytes and macrophages to help to eliminate bacteria and promote wound healing.

### 37.3.7 Interleukin-6

IL-6 is a pleiotropic cytokine. It has dual properties in proinflammation and anti-inflammation. Murine MSCs produce a high level of IL-6 even in resting status. Under inflammatory stimulation, IL-6 production can be further enhanced. When IL-6 is blocked by neutralizing antibody, it was demonstrated to be crucial in mediating the immunosuppression of MSCs on T cells [76]. Additionally, IL-6 produced by MSCs is important in suppressing the differentiation of bone marrow progenitors into dendritic cells [52].

### 37.3.8 Interleukin-10

Soon after the discovery of the immunosuppressive role of MSCs, IL-10 was found to mediate the suppressive effect of MSCs on T cell proliferation and Th17 cell differentiation. However, IL-10 production by non-stimulated MSCs does not occur, nor does it occur after stimulation with lipopolysaccharide/IL-3 [77–79]. Rather, IL-10 production in antigen-presenting cells such as dendritic cells and monocytes is upregulated when those cells interact with MSCs through cell–cell contact. Further investigation is required to determine whether such production of IL-10 is derived from MSCs or from the co-cultured immune cells. IL-10 blockade did not reverse MSC-mediated immunosuppression of lymphocytes [80].

### 37.3.9 Transforming growth factor-β

TGF-β is an immunosuppressive cytokine that counterbalances proinflammatory effects in a number of disease models. TGF-β has a direct effect in maintaining tissue homeostasis. It is a key factor in the induction of Tregs. Both *in vitro* and *in vivo* studies have demonstrated that TGF-β is important in mediating MSC suppression of T cells and NK cells in preclinical disease models including EAE, asthma and experimental autoimmune uveitis through Treg induction [57,81,82,83]. TGF-β secreted by MSCs can serve as a feedback loop to reverse the immunosuppression of MSCs on T cells via TGF-β receptors I and II, thus indicating a switching function of MSCs in converting from an immunosupressive role into an immune-enhancing one [84].

### 37.3.10 Human leukocyte antigen G

HLA-G is a nonclassical HLA class I molecule that is selectively expressed in cytotrophoblast cells at the maternal–fetal interface. It has been implicated in the mediation of maternal–fetal tolerance, since it is a ligand for inhibitory receptors of uterine NK cells. MSCs were recently reported to secrete a soluble (s) HLA-G5 isoform. Blockade of HLA-G5 reduces immunomodulatory functions of MSCs, including the *in vivo* expansion of $CD4^+CD25^+Foxp3^+$ Tregs, inhibition of T cell proliferation and suppression of NK-mediated cytolysis [16,85]. Thus HLA-G molecules play an important role in the modulation of immune response by MSCs.

### 37.3.11 Galectins

The human galectin family consists of 15 members. They are widely expressed by both immune cells and non-immune cells and play an important role in the maintenance of tissue homeostasis and regulation of the innate and adaptive immune responses. Galectin-1 has been shown to be a negative regulator of inflammation. It has two forms; one is soluble and the other is expressed on the cell surface membrane. Both forms can be detected in MSCs. Galectin-1 is released into the supernatant of MSCs in culture. Knockdown of galectin-1 on MSCs inhibits their immunosuppressive effect and restores T cell proliferation and IFN-γ production [86,87]. Galectin-9 has been reported to mediate an inhibitory role of MSCs in immune responses. On exposure to TLR ligands and inflammatory stimuli, MSCs can secrete galectin-9 to suppress T cell activation. Galectin-9 knockdown MSCs have a much lower immunosuppressive effect on mixed lymphocyte cultures than normal MSCs [88].

### 37.3.12 Other soluble immunosuppressive factors secreted by MSCs

Semaphorin-3A and leukemia inhibitory facor (LIF) are also involved in suppressing human T cell responses [87,89]. LL-37, an antimicrobial peptide secreted by MSCs, is upregulated by bacterial stimulation [90]. In a mouse model of *Escherichia coli* pneumonia, LL-37 secreted by MSCs increased bacterial clearance, suggesting that this is another paracrine effect of MSCs [90].

## 37.4 Conclusions

Investigations over the past several decades have dramatically increased our understanding of the potential therapeutic effects of MSCs. In addition to their function in cell replacement in certain circumstances, many studies have demonstrated that they produce a large number of soluble factors, especially in inflammation. They can orchestrate tissue repair by decreasing the effect of bacterial infection, by suppressing vigorous immune responses, and by promoting tissue regeneration. Further investigation of the potential mechanisms of these soluble factors is required. It would be desirable if paracrine factors alone could accomplish the same therapeutic effects as those of MSCs.

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## References

1 T.S. Li, M. Hayashi, H. Ito, *et al.* Regeneration of infarcted myocardium by intramyocardial implantation of *ex vivo* transforming growth factor-β-preprogrammed bone marrow stem cells. *Circulation* 2005;**111**:2438–2445.

2 Y. Miyahara, N. Nagaya, M. Kataoka, *et al.* Monolayered mesenchymal stem cells repair scarred myocardium after myocardial infarction. *Nat Med* 2006;**12**:459–465.

3 Y. Iso, J.L. Spees, C. Serrano, *et al.* Multipotent human stromal cells improve cardiac function after myocardial infarction in mice without long-term engraftment. *Biochem Biophys Res Commun* 2007;**354**:700–706.

4 R.H. Lee, A.A. Pulin, M.J. Seo, *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**:54–63.

5 K. Németh, A. Leelahavanichkul, P.S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

6 S.H. Mei, J.J. Haitsma, C.C. Dos Santos, *et al.* Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010;**182**:1047–1057.

7 B. Parekkadan, D. van Poll, K. Suganuma, *et al.* Mesenchymal stem cell-derived molecules reverse fulminant hepatic failure. *PLoS ONE* 2007;**2**:e941.

8 S.H. Mei, S.D. McCarter, Y. Deng, *et al.* Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 2007;**4**:e269.

9 L.A. Ortiz, M. Dutreil, C. Fattman, *et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of

mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A* 2007;**104**:11002–11007.

10 N. Gupta, X. Su, B. Popov, *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;**179**:1855–1863.

11 L.A. Ortiz, F. Gambelli, C. McBride, *et al.* Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**:8407–8411.

12 M. Yamada, H. Kubo, S. Kobayashi, *et al.* Bone marrow-derived progenitor cells are important for lung repair after lipopolysaccharide-induced lung injury. *J Immunol* 2004;**172**:1266–1272.

13 M. Rojas, J. Xu, C.R. Woods, *et al.* Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* 2005;**33**:145–152.

14 J. Xu, C.R. Woods, A.L. Mora, *et al.* Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *Am J Physiol Lung Cell Mol Physiol* 2007;**293**:L131–L141.

15 G. Zhen, H. Liu, N. Gu, *et al.* Mesenchymal stem cells transplantation protects against rat pulmonary emphysema. *Front Biosci* 2008;**13**: 3415–3422.

16 H. Choi, R.H. Lee, N. Bazhanov, *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood* 2011;**118**:330–338.

17 M. Rafei, P.M. Campeau, A. Aguilar-Mahecha, *et al.* Mesenchymal stromal cells ameliorate experimental autoimmune encephalomyelitis by inhibiting CD4 Th17T cells in a CC chemokine ligand 2-dependent manner. *J Immunol* 2009;**182**:5994–6002.

18 G. Ren, X. Chen, F. Dong, *et al.* Concise review: mesenchymal stem cells and translational medicine: emerging issues. *Stem Cells Transl Med* 2012;**1**:51–58.

19 P. Gao, Y. Zhou, L. Xian, *et al.* Functional effects of TGF-β1 on mesenchymal stem cell mobilization in cockroach allergen-induced asthma. *J Immunol* 2014;**192**:4560–4570.

20 Y. Wang, X. Chen, W. Cao, Y. Shi. Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nat Immunol* 2014;**15**:1009–1016.

21 H. Qian, H. Yang, W. Xu, *et al.* Bone marrow mesenchymal stem cells ameliorate rat acute renal failure by differentiation into renal tubular epithelial-like cells. *Int J Mol Med* 2008;**22**:325–332.

22 K.A. Cho, S.Y. Ju, S.J. Cho, *et al.* Mesenchymal stem cells showed the highest potential for the regeneration of injured liver tissue compared with other subpopulations of the bone marrow. *Cell Biol Int* 2009;**33**:772–777.

23 F. Han, C.Y. Wang, L. Yang, *et al.* Contribution of murine bone marrow mesenchymal stem cells to pancreas regeneration after partial pancreatectomy in mice. *Cell Biol Int* 2012;**36**:823–831.

24 R.A. Rose, H. Jiang, X. Wang, *et al.* Bone marrow-derived mesenchymal stromal cells express cardiac-specific markers, retain the stromal phenotype, and do not become functional cardiomyocytes *in vitro*. *Stem Cells* 2008;**26**:2884–2892.

25 D.J. Prockop, D.J. Kota, N. Bazhanov, R.L. Reger. Evolving paradigms for repair of tissues by adult stem/progenitor cells (MSCs). *J Cell Mol Med* 2010;**14**:2190–2199.

26 L. von Bahr, I. Batsis, G. Moll, *et al.* Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells* 2012;**30**:1575–1578.

27 F. Tögel, Z. Hu, K. Weiss, *et al.* Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 2005;**289**:F31–F42.

28 I. Sakaida, S. Terai, N. Yamamoto, *et al.* Transplantation of bone marrow cells reduces $CCl_4$-induced liver fibrosis in mice. *Hepatology* 2004;**40**:1304–1311.

29 L. Bai, D.P. Lennon, A.I. Caplan, *et al.* Hepatocyte growth factor mediates mesenchymal stem cell–induced recovery in multiple sclerosis models. *Nat Neurosci* 2012;**15**:862–870.

30 D.J. Prockop, J.Y. Oh. Mesenchymal stem/stromal cells (MSCs): role as guardians of inflammation. *Mol Ther* 2012;**20**:14–20.

31 D. Wolf, A. Reinhard, A. Seckinger, *et al.* Regenerative capacity of intravenous autologous, allogeneic and human mesenchymal stem cells in the infarcted pig myocardium-complicated by myocardial tumor formation. *Scand Cardiovasc J* 2009;**43**:39–45.

32 S.A. Eming, T. Krieg, J.M. Davidson. Inflammation in wound repair: molecular and cellular mechanisms. *J Invest Dermatol* 2007;**127**: 514–525.

33 A.D. Luster, R. Alon, U.H. von Andrian. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* 2005;**6**:1182–1190.

34 D.V. Krysko, G. Denecker, N. Festjens, *et al.* Macrophages use different internalization mechanisms to clear apoptotic and necrotic cells. *Cell Death Differ* 2006;**13**:2011–2022.

35 S. Ma, N. Xie, W. Li, *et al.* Immunobiology of mesenchymal stem cells. *Cell Death Differ* 2014;**21**:216–225.

36 Y. Shi, J. Su, A.I. Roberts, *et al.* How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol* 2012;**33**:136–143.

37 L. Chen, E.E. Tredget, P.Y. Wu, Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3**:e1886.

38 M. Gnecchi, H. He, O.D. Liang, *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* 2005;**11**:367–368.

39 L. Timmers, S.K. Lim, I.E. Hoefer, *et al.* Human mesenchymal stem cell-conditioned medium improves cardiac function following myocardial infarction. *Stem Cell Res* 2011;**6**:206–214.

40 X.L. Ma, K.D. Liu, F.C. Li, *et al.* Human mesenchymal stem cells increases expression of α-tubulin and angiopoietin 1 and 2 in focal cerebral ischemia and reperfusion. *Curr Neurovasc Res* 2013;**10**: 103–111.

41 S. Aguilar, C.J. Scotton, K. McNulty, *et al.* Bone marrow stem cells expressing keratinocyte growth factor via an inducible lentivirus protects against bleomycin-induced pulmonary fibrosis. *PLoS ONE* 2009;**4**:e8013.

42 S.P. Hung, M.H. Yang, K.F. Tseng, O.K. Lee. Hypoxia-induced secretion of TGF-β1 in mesenchymal stem cell promotes breast cancer cell progression. *Cell Transplant* 2013;**22**:1869–1882.

43 M. Giacca, S. Zacchigna. VEGF gene therapy: therapeutic angiogenesis in the clinic and beyond. *Gene Ther* 2012;**19**:622–629.

44 J.W. Lee, X. Fang, A. Krasnodembskaya, *et al.* Concise review: mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells* 2011;**29**:913–919.

45 W. Cao, Y. Yang, Z. Wang, *et al.* Leukemia inhibitory factor inhibits T helper 17 cell differentiation and confers treatment effects of neural

progenitor cell therapy in autoimmune disease. *Immunity* 2011;**35**:273–284.

46 A. Uccelli, L. Moretta, V. Pistoia. Mesenchymal stem cells in health and disease. *Nat Rev Immunol* 2008;**8**:726–736.

47 M.E. Bernardo, W.E. Fibbe. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013;**13**:392–402.

48 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.

49 A. Bartholomew, C. Sturgeon, M. Siatskas, *et al*. Mesenchymal stem cells suppress lymphocyte proliferation in vitro and prolong skin graft survival in vivo. *Exp Hematol* 2002;**30**:42–48.

50 E. Klyushnenkova, J. Mosca, K. McIntosh. Human mesenchymal stem cells suppress allogeneic T cell responses in vitro: implications for allogeneic transplantation. *Blood* 1998;**92**:2652.

51 P. Luz-Crawford, M. Kurte, J. Bravo-Alegría, *et al*. Mesenchymal stem cells generate a $CD4^+CD25^+Foxp3^+$ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem Cell Res Ther* 2013;**4**:65.

52 Y. Deng, S. Yi, G. Wang, *et al*. Umbilical cord-derived mesenchymal stem cells instruct dendritic cells to acquire tolerogenic phenotypes through the IL-6-mediated upregulation of SOCS1. *Stem Cells Dev* 2014;**23**:2080–2092.

53 K. Akiyama, C. Chen, D. Wang, *et al*. Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012;**10**:544–555.

54 M.H. Abumaree, M.A. Al Jumah, B. Kalionis, *et al*. Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages. *Stem Cell Rev* 2013;**9**: 620–641.

55 K. Le Blanc, D. Mougiakakos. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol* 2012;**12**:383–396.

56 X.X. Jiang, Y. Zhang, B. Liu, *et al*. Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**:4120–4126.

57 P.A. Sotiropoulou, S.A. Perez, A.D. Gritzapis, *et al*. Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**:74–85.

58 G.M. Spaggiari, A. Capobianco, S. Becchetti, *et al*. Mesenchymal stem cell–natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;**107**:1484–1490.

59 A. Corcione, F. Benvenuto, E. Ferretti, *et al*. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**:367–372.

60 G. Ren, L. Zhang, X. Zhao, *et al*. Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**:141–150.

61 M. Krampera, L. Cosmi, R. Angeli, *et al*. Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**:386–398.

62 G. Ren, J. Su, L. Zhang, *et al*. Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells* 2009;**27**:1954–1962.

63 R.M. Bingisser, P.A. Tilbrook, P.G. Holt, U.R. Kees. Macrophage-derived nitric oxide regulates T cell activation via reversible disruption of the Jak3/STAT5 signaling pathway. *J Immunol* 1998;**160**:5729–5734.

64 W. Li, G. Ren, Y. Huang, *et al*. Mesenchymal stem cells: a double-edged sword in regulating immune responses. *Cell Death Differ* 2012;**19**:1505–1513.

65 R. Meisel, A. Zibert, M. Laryea, *et al*. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**:4619–4621.

66 A.L. Mellor, D.H. Munn. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat Rev Immunol* 2004;**4**:762–774.

67 M. François, R. Romieu-Mourez, M. Li, J. Galipeau. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther* 2012;**20**:187–195.

68 G.M. Spaggiari, A. Capobianco, H. Abdelrazik, *et al*. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008;**111**:1327–1333.

69 C. Bouffi, C. Bony, G. Courties, *et al*. IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. *PLoS ONE* 2010;**5**:e14247.

70 G.M. Spaggiari, H. Abdelrazik, F. Becchetti, L. Moretta. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin E2. *Blood* 2009;**113**:6576–6583.

71 J.H. Ylöstalo, T.J. Bartosh, K. Coble, D.J. Prockop. Human mesenchymal stem/stromal cells cultured as spheroids are self-activated to produce prostaglandin E2 that directs stimulated macrophages into an anti-inflammatory phenotype. *Stem Cells* 2012;**30**:2283–2296.

72 K. English, J.M. Ryan, L. Tobin, *et al*. Cell contact, prostaglandin $E_2$ and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of $CD4^+CD25^{High}$ forkhead box $P3^+$ regulatory T cells. *Clin Exp Immunol* 2009;**156**: 149–160.

73 M. Matysiak, W. Orlowski, M. Fortak-Michalska, *et al*. Immunoregulatory function of bone marrow mesenchymal stem cells in EAE depends on their differentiation state and secretion of PGE2. *J Neuroimmunol* 2011;**233**:106–111.

74 H. Denney, M.R. Clench, M.N. Woodroofe. Cleavage of chemokines CCL2 and CXCL10 by matrix metalloproteinases-2 and -9: implications for chemotaxis. *Biochem Biophys Res Commun* 2009;**382**:341–347.

75 M. Rafei, J. Hsieh, S. Fortier, *et al*. Mesenchymal stromal cell-derived CCL2 suppresses plasma cell immunoglobulin production via STAT3 inactivation and PAX5 induction. *Blood* 2008;**112**:4991–4998.

76 F. Djouad, L.M. Charbonnier, C. Bouffi, *et al*. Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 2007;**25**:2025–2032.

77 S. Beyth, Z. Borovsky, D. Mevorach, *et al*. Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;**105**:2214–2219.

78 M.E. Groh, B. Maitra, E. Szekely, O.N. Koç. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Exp Hematol* 2005;**33**:928–934.

79 I. Rasmusson, O. Ringdén, B. Sundberg, K. Le Blanc. Mesenchymal stem cells inhibit lymphocyte proliferation by mitogens and alloantigens by different mechanisms. *Exp Cell Res* 2005;**305**:33–41.

80 G. Xu, L. Zhang, G. Ren, *et al*. Immunosuppressive properties of cloned bone marrow mesenchymal stem cells. *Cell Res* 2007;**17**:240–248.

81 M.O. Li, R.A. Flavell. Contextual regulation of inflammation: a duet by transforming growth factor-β and interleukin-10. *Immunity* 2008;**28**:468–476.

82 R. Tasso, C. Ilengo, R. Quarto, *et al*. Mesenchymal stem cells induce functionally active T-regulatory lymphocytes in a paracrine fashion and ameliorate experimental autoimmune uveitis. *Invest Ophthalmol Vis Sci* 2012;**53**:786–793.

83 K. Nemeth, A. Keane-Myers, J.M. Brown, *et al*. Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci U S A* 2010;**107**: 5652–5657.

84 C. Xu, P. Yu, X. Han, *et al*. TGF-β promotes immune responses in the presence of mesenchymal stem cells. *J Immunol* 2014;**192**:103–109.

85 R. Rizzo, D. Campioni, M. Stignani, *et al*. A functional role for soluble HLA-G antigens in immune modulation mediated by mesenchymal stromal cells. *Cytotherapy* 2008;**10**:364–375.

86 F. Gieseke, J. Böhringer, R. Bussolari, *et al*. Human multipotent mesenchymal stromal cells use galectin-1 to inhibit immune effector cells. *Blood* 2010;**116**:3770–3779.

87 Y. Lepelletier, S. Lecourt, A. Renand, *et al*. Galectin-1 and semaphorin-3A are two soluble factors conferring T-cell immunosuppression to bone marrow mesenchymal stem cell. *Stem Cells Dev* 2010;**19**:1075–1079.

88 F. Gieseke, A. Kruchen, N. Tzaribachev, *et al*. Proinflammatory stimuli induce galectin-9 in human mesenchymal stromal cells to suppress T-cell proliferation. *Eur J Immunol* 2013;**43**:2741–2749.

89 A. Nasef, C. Mazurier, S. Bouchet, *et al*. Leukemia inhibitory factor: role in human mesenchymal stem cells mediated immunosuppression. *Cell Immunol* 2008;**253**:16–22.

90 A. Krasnodembskaya, Y. Song, X. Fang, *et al*. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010;**28**:2229–2238.

CHAPTER 38

# Treatment of lung disease by mesenchymal stromal cell extracellular vesicles

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## 38.1 Introduction

Mesenchymal stromal cells (MSCs) may potentially be an innovative treatment for acute and chronic lung disease owing to their ability to secrete paracrine factors such as growth factors, anti-inflammatory cytokines, and antimicrobial peptides [1–9]. This is in addition to the "immuno-privileged" property of MSCs allowing allogeneic transplantation and lack of evidence of tumor formation *in vivo*. Based on significant preclinical data, multiple phase I and II clinical trials (listed in ClinicalTrial.gov) have been performed or are ongoing in a variety of acute and chronic lung diseases, such as acute respiratory distress syndrome (ARDS; NCT01775774, NCT01902082, NCT02215811), severe bronchopulmonary dysplasia (NCT01207869, NCT01297205), asthma (NCT02192736), chronic obstructive pulmonary disease (COPD; NCT00683722), emphysema (NCT01849159), and

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idiopathic pulmonary fibrosis (NCT02135380). However, concerns still remain as to whether MSCs can become tumorigenic or promote preexisting tumors, such as breast cancer [10–12].

Recently, MSCs have been found to release extracellular vesicles such as exosomes and microvesicles (MV) that are as biologically active as the cells themselves in a variety of inflammatory syndromes. In this chapter we review the current preclinical studies on the use of exosomes or MVs derived from MSCs as a therapeutic agent in both acute and chronic lung diseases.

## 38.2 Definitions and characterization of extracellular vesicles

Once considered cellular debris or dust, increasing evidence now demonstrates that secreted extracellular vesicles play important roles in biology (e.g., in intercellular traffic and communication) and in disease (e.g., in inflammation or cancer). Multiple cell types, including but not limited to platelets, megakaryocytes, monocytes, epithelial and endothelial cells, lymphocytes, polymorphonuclear leukocytes, stem cells, and cancer cells, secrete vesicles following diverse physiological or pathophysiological processes or triggers or in response to external stimuli. Vesicles can be found in blood, urine, organs, and tissues or cavities such as the alveolar space. Currently, research into the biological importance of vesicles ranges from potential biomarkers of disease to mechanisms for injury or repair in part through the transfer of proteins or peptides, mRNA, microRNA, and/or organelles contained in the vesicles of the targeted tissue [13,14].

However, owing to the ubiquitous nature of vesicles in normal physiology and pathophysiology, there is no broad agreement on classification or nomenclature of vesicles. In the first meeting of the International Society of Extracellular Vesicles in 2012 no

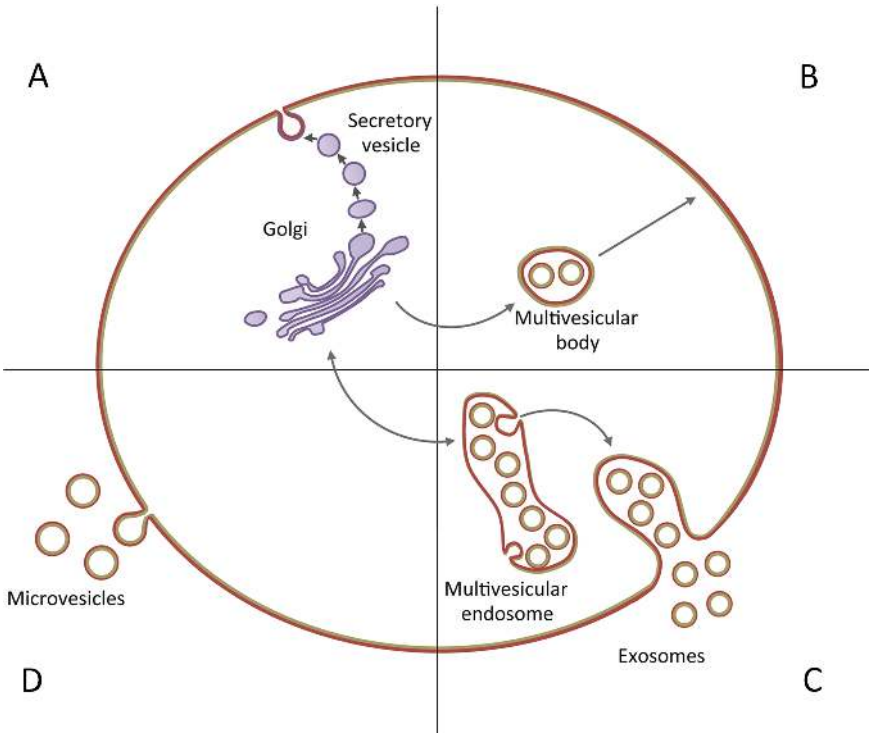



**Figure 38.1** Extracellular vesicles: diversity of origins and mechanisms of interactions with recipient cells.
Extracellular vesicles originate from different subcellular compartments. (A) Intracellular trafficking toward the plasma membrane for secretion of soluble components (e.g., proteins, lipids, RNA) occurs via secretory vesicles that contain intraluminal components. (B, C) Alternatively, extracellular vesicles can form inside internal compartments such as early endosomes, through the invagination of the endosomal membrane to generate intraluminal vesicles within either multivesicular bodies (B) or multivesicular endosomes (C). The intraluminal vesicles are then released by fusion of these compartments with the plasma membrane. Vesicles generated in either multivesicular bodies or endosomes are called exosomes once secreted. (D) By contrast, secreted extracellular vesicles contain cytoplasmic components. Secreted extracellular vesicles can form at the plasma membrane by direct budding into the extracellular space, giving rise to microvesicles and membrane particles.
Mechanisms of interactions between extracellular vesicles and recipient cells. (E) Extracellular vesicles can deliver their bioactive cargo through the fusion-mediated transfer of surface receptors, proteins, lipids, and genetic material (e.g., DNA and RNA). (F) They can also initiate transduction of a cell-signaling pathway through either a ligand–receptor interaction or (G) through a paracrine effect from a secreted factor. (H) Lastly, internalization and lysosomal processing of extracellular vesicles represents another mechanism of interaction, which can lead to both the expression of the content of the extracellular vesicles on the surface of the recipient cell and the delivery of their bioactive cargo.

consensus was reached on vesicle nomenclature, but a general agreement was made "that investigators should not be forced to concede their scientific independence" [15]. In an attempt to cope with "imperfect nomenclature," Gould and Raposo recommended that scientists should state their use of the terms for vesicles explicitly based on precedent or logical argument and the method of collection of the vesicles in their publications [16].

## 38.3 Nomenclature defined by size and morphology

For research purposes, vesicles can now be broadly defined by their size and morphology as exosomes, MVs, and apoptotic bodies. Exosomes are vesicles 50–100 nm in size that arise from endosomal membrane compartments and are released into the extracellular space after fusion of multivesicular bodies with the cell membrane [17–21]. Exosomes are rich in annexins, tetraspanins (CD63, CD81, CD9), and heat-shock proteins (Hsp60, Hsp70, and Hsp90) and express low amounts of phosphatidylserine. In addition, tumor susceptibility gene 101 (Tsg101), Alix, and clathrin are frequently present in exosomes. More importantly, exosomes carry proteins and RNAs unique to their cell source and their pathophysiological state [22,23]. The list of proteins and RNAs reported to be present in exosomes is freely accessible at Exocarta [24] (http://www.exocarta.org) or Vesiclepedia [25] (http://www.microvesicles.org).

MVs (often referred to as ectosomes, microparticles, or shed vesicles/particles) originate by direct budding off the plasma membrane and range in size from 100 to 1000 nm [26–28]. These vesicles express high amounts of phosphatidylserine, contain proteins associated with lipid rafts, and are enriched in cholesterol, sphingomyelin, and ceramide.

Apoptotic bodies (also called apobodies) are larger sealed membrane vesicles that are produced from cells undergoing programmed cell death and are generally >800 nm in size. The formation of apoptotic bodies is a mechanism by which dying cells prevent leakage of potentially toxic or immunogenic cellular content, preventing inflammation and tissue destruction, by coordinated uptake by phagocytes. In contrast, exosomes and MVs are constitutively released and can be induced by multiple cell types and often serve as a means of intercellular communication, whether through normal physiological processes or in response to injury (Figure 38.1).

Both exosomes and MVs may communicate with target cells by specific receptor–ligand interactions and transfer receptors and biologically active molecules to these target cells following internalization [29]. Moreover, besides proteins and lipids, both exosomes and MVs also carry mRNA and microRNA and thus may play a role in the exchange of genetic material between cells [27–30].

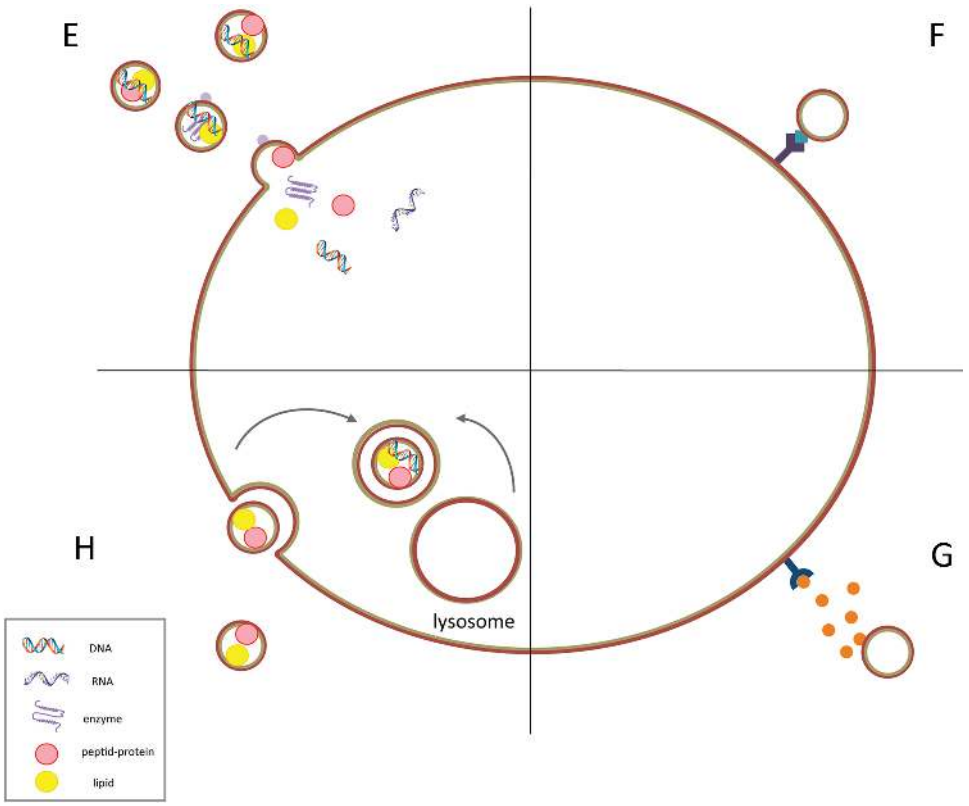



**Figure 38.1** (*Continued*)

## 38.4 Common methods of collection of extracellular vesicles

Common methods used to isolate extracellular vesicles include techniques such as ultracentrifugation, filtration, and immunoaffinity isolation [15].

### 38.4.1 Ultracentrifugation

Vesicle isolation by differential centrifugation is the most commonly used technique utilized in the literature. Body fluid samples such as plasma or serum or from culture medium are depleted first of cells, platelets, and large apoptotic bodies by low-speed centrifugation steps and/or size exclusion filters (e.g., 800 nm filters). Forces commonly reported for this step are in the 10 000–20 000*g* range. Smaller vesicles are then pelleted at higher speeds (100 000–120 000*g*). However, these sequential ultracentrifugation steps cannot achieve absolute separation of vesicles by size because sedimentation also depends on the density of the vesicle and the distance a vesicle travels. Vesicle pellets from high-speed ultracentrifugation will also contain extravascular protein complexes or aggregates, lipoprotein particles, and other contaminants. Although used frequently to quantitate vesicles (as discussed later), protein concentration measurement of vesicles may be inaccurate due to these contaminations. To separate contaminating protein and/or protein–RNA aggregates from the lipid-containing vesicles, density sucrose gradients or sucrose cushions are used to float the relatively low-density vesicles, classically reported in the range between 1.1 and 1.19 g/mL, away from other contaminants. Although density separation is more precise, such separation can require centrifugation of a sample for up to 90 h. Consequently, when reporting methods utilized to isolate the vesicles, it is important to specify the *g* force, rotor type (fixed angle, swinging bucket), pelleting efficiency (rotor *k*-factor), sample viscosity, and gradient characteristics.

### 38.4.2 Size exclusion

Using filters, separation of vesicles based on size can be achieved. Filters of 0.8 μm are often used to remove large cell fragments and platelets prior to vesicle isolation, while 0.2 μm filters may be used for the isolation of smaller vesicles. However, some concerns remain that forcing vesicles through filter pores may cause deformation and breakup of larger particles or platelets, thereby changing the characteristics of the recovered vesicles. To prevent this, size exclusion should be performed by gravity. Column chromatography may allow sequential elution of vesicles of different size fractions from a single column. However, filter-based methods will not enrich vesicle populations unless low molecular weight filters (e.g., Centricon) are used for concentration.

### 38.4.3 Immunoaffinity isolation

The presence of characteristic surface proteins on certain vesicles is the basis for immunoaffinity isolation in which antibodies to surface proteins are used to select desired vesicle populations (immuno-enrichment) or to trap unwanted vesicle populations (immuno-depletion). Antibodies are attached to beads or other matrices through covalent or high-affinity interactions, facilitating physical separation by low-speed centrifugation or magnetic techniques. Immunologic techniques have the potential for high specificity of the collected vesicles. However, high selectivity may result in lower yields of the vesicles because not all vesicles will express the marker or protein of interest.

### 38.4.4 Polymeric precipitation

Commercial kits containing polymeric precipitation mixtures are incubated with the fluid containing the vesicles overnight and low-speed ultracentrifugation is then used to collect the precipitate containing vesicles. By tying up water molecules, the reagent in theory forces less-soluble components, such as vesicles, out of solution, which allows them to be collected by low-speed centrifugation. However, as with ultracentrifugation, isolation of vesicles by polymer-based precipitation methods may include numerous contaminants, such as lipoproteins. In general, this technique may produce a higher yield of RNA at the cost of inclusion of impurities.

Research studies into exosomes or MVs derived from MSCs often use ultracentrifugation and size exclusion filters to isolate the vesicles.

## 38.5 Quantification of extracellular vesicles

Several methods have been developed to quantify vesicles from different preparation methods. Often, the major limitation to the quantification technique used by investigators is the cost or lack of availability of the instruments required.

### 38.5.1 Optical single-particle tracking: nanoparticle tracking analyses

Nanoparticle tracking analyses (NTA) is the commercial name of an optical particle tracking method. In this method, a laser beam is scattered by particles in the sample of interest and the mean velocity of each particle is calculated by the Stokes–Einstein equation on the basis of Brownian motion as recorded by a charge-coupled-device camera. This technique is used to obtain the concentration and size distribution of vesicle populations. Prior to use, vesicles should be isolated from the fluid of interest in order to remove contaminating lipoprotein particles, protein complexes, and other particles of similar size. Although standard light scatter NTA does not detect the cellular origin of vesicles, such analyses can be made using fluorescent-labeled antibodies to surface proteins on vesicles of interest. It may be useful to compare samples treated with and without detergents to eliminate the vesicles to check for the amount of debris in the sample.

### 38.5.2 Flow cytometry

Conventional flow cytometry has a lower detection threshold around 500 nm and is suited for analysis of large vesicles. Newer instruments, such as Gallios (Beckman Coulter), BD-Influx or LSR Fortessa (Becton Dickinson), and Apogee (Apogee Flow Systems), can discriminate particles as small as 100–200 nm. The International Society on Thrombosis and Haemostasis Scientific Standardization Committee proposed a protocol that uses 500 nm and 900 nm polystyrene beads from Megamix to define a vesicle size gate. However, even with the use of appropriate standards such as polystyrene beads or silica particles to quantitate the number of vesicles, it remains difficult by flow cytometry to distinguish single vesicles from "swarm" detection (i.e., multiple vesicles are simultaneously illuminated by the laser beam and counted as a single event), underestimating the concentration of vesicles [31]. The current recommendation is to analyze serial dilutions of the vesicle sample to define the proper dilution at which "swarm" signals are minimized. As with NTA, detergents can be used to estimate the amount of debris contaminating the sample. Recently, investigators have used fluorescent-labeled antibodies to surface receptors such as CD44 and CD29 on vesicles to determine the concentration. Although specific enough to quantitate the number of fluorescent hits, the technique in general may underestimate the true number of vesicles due to low to moderate sensitivity of the surface protein on the vesicle.

### 38.5.3 Electron microscopy

Using heavy metal stains such as osmium tetroxide and uranyl acetate, transmission electron microscopy has been used to characterize membrane-bound vesicles in cells and in isolation of pure vesicles. Transmission electron microscopy has also been used with immunoglobulin coupled to nanogold particles that detect specific vesicle features (immuno-EM). Electron microscopy is valuable for the assessment of morphology, size, and presence of markers (immuno-EM). However, the technique is of limited use for the determination of concentration of vesicles.

### 38.5.4 Protein concentration

Protein concentration using a standard protein assay (i.e., Bradford) for both the membrane and intracellular components of the vesicles has been used to quantitate and compare vesicles from different isolation techniques. However, the use of protein concentration will not reveal the number of cells from which the vesicles were released and may also be affected by any preconditioning steps the cells undergo prior to the release of the vesicles.

### 38.5.5 Cell count

For *in vitro* experiments, preconditioning the cells of interest (e.g., by serum starvation, inflammation, or hypoxia) prior to isolation of the released vesicles can dramatically change the phenotype and the surface and/or intracellular compositions of the vesicles released. An example is vesicles derived from M1 or M2 macrophages. Consequently, many investigators have quantified the number of vesicles by the number of cells from which they are derived and the preconditioning steps used to release the vesicles. Benefits of using cell count to quantify the vesicles are (1) the ability to compare the released vesicles with the parent cells as in cell-based therapy and (2) the lack of need for expensive equipment.

Research studies into exosomes or MVs derived from mesenchymal stromal cells have used all of these techniques equally.

## 38.6 Interaction of extracellular vesicles with targeted cells

Extracellular vesicles may influence the behavior of recipient cells by several different mechanisms. First, vesicles may act as signaling complexes by direct stimulation of target cells. Vesicles may express several types of receptors and surface molecules, such as tissue factor (TF), tumor necrosis factor (TNF), major histocompatibility complex class I/II molecules, chemokine receptor CCR5 [32], intercellular adhesion molecule 1 (ICAM-1) [33], and delta-like ligand 4 [34]. These molecules may directly activate cells bearing the respective ligands and/or transfer receptors and/or bioactive lipids between cells after fusion with the target cell membrane [35]. For MVs derived from MSCs, Camussi and colleagues found that the membrane receptors CD44 and CD29 were critical for targeting MSC MVs to injured renal epithelium in a mouse model of acute kidney injury (AKI). Prior trypsinization of the MSC MVs eliminated trafficking toward the targeted cell and the therapeutic effects of the MVs [36].

Second, vesicles may modulate target cells by delivering their intracellular protein cargo. For example, vesicles can activate angiogenesis through the transfer of proangiogenic molecules such as vascular endothelial growth factor, basic fibroblast growth factor, TNF-α, and transforming growth factor (TGF)-β [37] along with proteases such as matrix metalloproteases 2/9 and membrane type 1 metalloprotease [38] and their respective activators [39]. Exosomes can reverse the depletion of key enzymes such as peroxiredoxins and glutathione *S*-transferase involved in reactive oxygen species metabolism and reduce the resultant accumulation of toxic products from the electron transport chain by direct transfer into injured cells during oxidative stress-induced injury [40]. Exosomes also contain key enzymes involved in the adenosine-5′-triphosphate (ATP)-generating stage of glycolysis, potentially restoring proteomic alterations in injured tissues [41,42]. For MVs derived from MSCs, Islam *et al.* found that one of the therapeutic effects of MSC MVs was through the transfer of mitochondria to the injured alveolar epithelium with subsequent restoration of ATP levels in a mouse model of acute lung injury [43]. In contrast to a beneficial biological effect, vesicles can also induce apoptosis in target cells by transferring caspase-1, caspase-8, or lymphotoxin-α [44].

Third, vesicles can transfer mRNAs and small noncoding RNA species between cells as a mechanism for cell-to-cell communication [45]. It has become evident that vesicles contain a distinct cargo that not only represents the cell of origin but also are enriched in specific nucleic acid or lipid species that can be transferred to target cells and subsequently translated into proteins [30,46]. In several studies, pretreatment of vesicles with RNase inhibited the observed biological effects of the vesicles, demonstrating the important contributions of vesicle-derived RNA [27,28]. These nucleic acids can be transferred to recipient cells and induce functional/phenotypic changes in the recipient cells [47,48]. Zhu *et al.* found that one of the therapeutic properties of MSC MVs was through the transfer of mRNA for keratinocyte growth factor to the injured alveolus with subsequent expression of the protein in a mouse model of acute lung injury [14] (Figure 38.1).

## 38.7 Endogenous extracellular vesicles in lung disease

Occupying a pivotal interface between the internal milieus of the body with the external environment, the lung is a unique organ composed of multiple cellular lineages forming its parenchyma and circulating within the alveolar space. Among the cells that occupy the alveolus are epithelial cells, endothelial cells, fibroblasts, monocytes and macrophages, polymorphonuclear neutrophils (PMNs), dendritic cells, and lymphocytes. There is intense cell-to-cell communication to maintain homeostasis or to become involved in inflammatory lung diseases such as ARDS, asthma, and COPD. Interestingly, most cell types have been shown capable of secreting in their environment a wide array of extracellular vesicles, ranging from exosomes to the larger MVs [49]. Far from "cell garbage" as originally thought [50], released extracellular vesicles are *sine qua non* (essential) for cell-to-cell communication, which in turn is critical for homeostasis and for generating an efficient response to lung injury [13]. In the following sections we will provide a brief overview of the main characteristics of endogenous extracellular vesicles and review the available body of knowledge on their role in ARDS, COPD, and asthma, describing both their detrimental and beneficial effects in lung injury. Then we will demonstrate how endogenous extracellular vesicles may serve as biomarker in lung disease, potentially representing a therapeutic target (Figure 38.2).

### 38.7.1 Endogenous extracellular vesicles in acute respiratory distress syndrome

Clinically, ARDS, the most common cause of respiratory failure in intensive care units, is defined by bilateral chest infiltrates without evidence of left heart failure and a $PaO_2/FIO_2$ ratio <300 [51]. Depending on the etiology, pneumonia and sepsis being most the common, the mortality rate can reach 40%. The pathophysiology of ARDS involves complex crosstalk between both the immune and coagulation systems with the alveolo-capillary lung barrier, leading to an excessive proinflammatory response, increase in lung protein permeability, and formation of pulmonary edema, which eventually results in impaired gas exchange and hypoxemia [52]. A growing body of research has revealed the critical role of extracellular vesicles as a means of communication between the multiple different cells involved in ARDS.

Extracellular vesicles released by platelets (PEVs) have been shown to interact with key players in ARDS, leading to the activation of endothelial cells, PMNs, and platelets themselves [53–55]. Exposure to PEVs was found to stimulate lung endothelial cells to secrete proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-8, monocyte chemoattractant protein-1 (also known CCL2), monocyte-derived IL-1, TNF-α, and IL-8. In addition, IL-1β-enriched PEVs increased the expression of vascular cell adhesion molecule-1 (VCAM-1) on the endothelium [53]. Interaction between PEVs and PMNs appeared to be mediated by different ligand–receptor linkage, such as the platelet glycoprotein Ib–the leukocyte interin Mac-1, β2-integrins (CD11b)–Mac-1, or P-selectin–P-selectin glycoprotein ligand-1, promoting IL-8 secretion, oxidative burst, degranulation, and enhanced leukocyte rolling [54,55]. The arachidonic acid (AA)–thromboxane A2 (TXA2) signaling pathway largely underlies the proinflammatory properties of PEVs. PEVs activated by either soluble phospholipase A2 or ExoU (a *Pseudomonas aeruginosa* toxin with phospholipase A2 activity), converts the newly formed AA to TXA2, promoting platelet activation and, more importantly, resulting in a hypercoagulable state with increased lung protein permeability [56,57]. Overall, these studies demonstrated that platelets, monocytes, and endothelial cells were activated through either AA or one of its downstream prostanoid metabolites released by PEVs [58–60]. As with PEVs, PMN extracellular vesicles (NEVs) were also found to stimulate platelets, endothelial cells, and other immune cells directly or indirectly through platelet-activating factor secretion. Multiple studies have demonstrated the capacity of NEVs to stimulate endothelial cells to increase IL-6 secretion, leading to vascular dysfunction by altering the balance between nitric oxide and reactive oxygen species production, which results in oxidative stress [61–63]. Injured endothelial cells in turn subsequently release extracellular vesicles, which further modulates inflammation and coagulation.

Coagulation disorders characterized by hypercoagulability and hypofibrinolytic states are key features of ARDS [52,64]. TF (also known as platelet TF, factor III, thromboplastin, and CD142), which is a key initiator in the coagulation cascade, is widely expressed by diverse extracellular vesicles in ARDS [65,66] such as PEVs [67,68] and is critically involved in the coagulation abnormality. While some extracellular vesicles (such as those released by monocytes) decrease the expression of the TF pathway inhibitor and thrombomodulin (which promotes thrombogenicity *in vitro* [69]), most of the procoagulant effect is mediated through TF extracellular vesicles during inflammation [65,70]. In addition, the large amount of

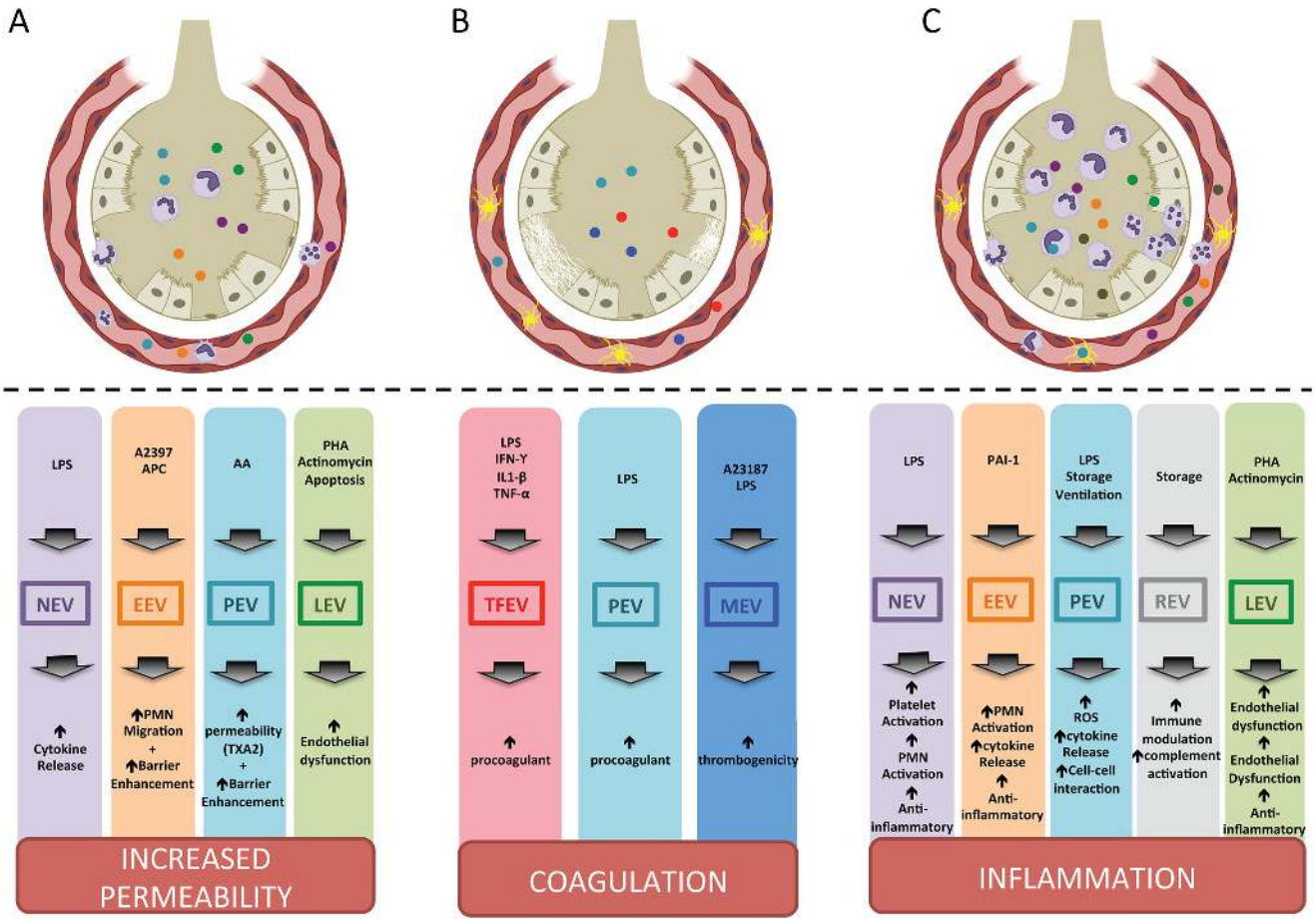



**Figure 38.2** Schematic representation of endogenous extracellular vesicles and their involvement in acute lung injury. The pathological effects of endogenous extracellular vesicles in acute lung injury are depicted under three major pathways: increased lung protein permeability (A), a hypercoagulable state (B), and inflammation (C). Extracellular vesicles (color coded) released by specific cells are capable of impacting these three pathways of lung injury at multiple levels. As described further in the text, the different extracellular vesicle populations are in turn activated or released upon specific stimuli. (See color plate section.)
AA: arachidonic acid; APC: activated protein C; EEV: endothelial extracellular vesicles (orange); IFN: interferon; IL-1: interleukin 1; LEV: lymphocyte extracellular vesicles (green); LPS: lipopolysaccharide; MEV: monocyte extracellular vesicles (dark blue); NEV: neutrophil extracellular vesicles (purple); PAI-1: plasminogen activator inhibitor 1; PEV: platelet extracellular vesicles (light blue); PHA: phytohemagglutinin; PMN: polymorphonuclear neutrophil; REV: red blood cell extracellular vesicles (grey); ROS: reactive oxygen species; TFEV: tissue factor expressing extracellular vesicles (red); TNF: tumor necrosis factor; TXA2: thromboxane A2.

phosphatidylserine present on the surface as well as the amount of thrombin and von Willebrand factor multimers expressed and released by extracellular vesicles also contribute to the procoagulant effects during inflammation [71–73]. In a study comparing bronchoalveolar lavage (BAL) fluid from ARDS patients with patients with cardiogenic pulmonary edema, TF-enriched extracellular vesicle concentration was significantly higher in the BAL fluid of ARDS patients [74]. Lastly, aside from platelets, alveolar epithelial cells can also generate procoagulant extracellular vesicles in inflammation [74,75].

Disruption of alveolar epithelial and endothelial barriers, leading to altered lung protein permeability, is another hallmark of ARDS. As described earlier, TXA2, which is one of the mediators responsible for an increase in lung vascular permeability [76,77], can be generated and released by PEVs, especially during inflammation. A direct link between high levels of circulating PEVs and TXA2 with increased lung vascular permeability was shown in infection-induced acute lung injury in mice [57]. Moreover, several studies clearly demonstrated that extracellular vesicles released by endothelial cells upon inflammation altered local nitric oxide production, leading to increased barrier permeability [71]. This capacity of endothelial-cell-derived extracellular vesicles (EEVs) to induce lung injury through the alteration of endothelial function has been found in several additional *in vivo* acute lung injury animal models [78–80]. Interestingly, although in these studies investigators used EEVs produced from human lung endothelial cells exposed to either physiologic or mechanical stress or to molecular triggers such as lipopolysaccharide or plasminogen activator inhibitor 1, extracellular vesicles released by direct acute lung injury

produced similar features, such as neutrophil infiltration with increased lung vascular permeability and proinflammatory cytokines release. Overall, these studies demonstrated that extracellular vesicles from diverse cell types during injury are able to promote inflammation, a hypercoagulable state, and endothelial dysfunction, which can lead to ARDS.

Clinically, however, patients with higher levels of circulating leukocyte extracellular vesicles or EEVs have been associated with better outcomes with a lower mortality rate in both sepsis and ARDS [81,82], suggesting a more complex role of these extracellular vesicles in lung injury. This important finding demonstrating some beneficial advantages from higher extracellular vesicles deserves further investigation. For example, PEVs have been found to express lipoxygenase 12, which once transferred into mast cells promotes synthesis and secretion of lipoxin $A_4$ [83], a factor known to exert anti-inflammatory properties that are able to attenuate acute lung injury [84,85]. Other investigators have shown the capacity of NEVs to express annexin A1 [86] or to stimulate the secretion of TGF-β [87], both of which have anti-inflammatory properties and can also exert increased bactericidal activity [88]. Timár *et al.* found that neutrophils prestimulated with opsonized particles were able to release extracellular vesicles that exerted increased bactericidal activity through cytoskeletal reorganization and impairment of bacterial growth. These effects might contribute to their anti-inflammatory effect in lung injury induced by infection. Concerning the impact of extracellular vesicles on coagulation in ARDS, studies have found that extracellular vesicles can also exhibit anticoagulant properties through protease-activated receptor-1 and -2 signaling pathways [89] and increased expression of anticoagulant factors such as thrombomodulin and protein S [90]. Finally, although extracellular vesicles have been associated with endothelial dysfunction in ARDS, some authors have demonstrated that extracellular vesicles carrying sonic hedgehog protein might be beneficial for the endothelium by increasing nitric oxide release and reducing reactive oxygen species production [91–93].

In summary, endogenous extracellular vesicles released by diverse cell types are involved in the pathophysiology of ARDS and are able to exert both detrimental and beneficial effects on inflammation, coagulation, and endothelial function. Thus, any strategy aiming at using extracellular vesicles as a potential therapeutic target will need to identify the phenotype of the released extracellular vesicles first.

### 38.7.2 Endogenous extracellular vesicles in chronic obstructive pulmonary disease

COPD is characterized by progressive destruction of lung parenchyma, resulting in airflow reduction [94]. A growing body of evidence indicates that cell injury, increased apoptosis, and accelerated senescence of both epithelial and endothelial cells contribute significantly to the lung destruction encountered in COPD [95–101]. Since the release of extracellular vesicles constitutes an important response of endothelial cells to injury, EEVs have represented the main focus of the majority of studies in COPD. Eight cell surface markers have been found to be expressed on the surface of EEVs in COPD [102]. They are vascular endothelial-cadherin (CD144), platelet endothelial cell adhesion molecule-1 (CD31), melanoma cell adhesion molecule (CD146), E-selectin (CD62E), integrin $\alpha_V$ chain (CD51), endoglin (CD105), von Willebrand factor, and angiotensin-converting enzyme (CD143). Based on preclinical evidence, EEVs in COPD appear to be involved in endothelial junction preservation or disruption [103], platelet function, angiogenesis, T and B cell activation, endothelial cell permeability and signal transduction, transmigration across the endothelium, and leukocyte recruitment to the site of injury [104–110]. Unlike extracellular vesicles in ARDS described earlier, which can both exacerbate and suppress inflammation, EEVs appear to primarily propagate inflammatory injury. However, further studies investigating the impact of EEVs released in COPD on other cells are needed. Notably, to our knowledge, no studies have been carried out on the biological cargo of EEVs (such as mRNA, microRNA, or proteins), the impact of which on the epithelium, endothelium, and immune cells involved in COPD may be important. Nevertheless, smoking exposure [111–113], oxidative stress [113], and infection [114–117] are known to trigger EEV release, suggesting an involvement of EEVs in an early stage of the mechanisms underlying the disease. In addition, two different research teams have demonstrated a differential pattern of EEV release, enabling the distinction of COPD patients from non-COPD healthy volunteers as well as demonstrating different levels at different stages of the disease [118–120]. These studies showed first that in EEVs the levels of circulating vascular endothelial-cadherin-, platelet endothelial cell adhesion molecule-1-, and E-selectin-positive were higher in stable COPD patients than in non-COPD patients [118]. Second, they showed that the levels of circulating vascular endothelial-cadherin-, platelet endothelial cell adhesion molecule-1-, and E-selectin-positive EEVs were increased during exacerbations of COPD compared with stable COPD [118]. Third, they showed that even patients with a mild or moderate COPD had a higher systemic level of platelet endothelial cell adhesion molecule-1-positive EEVs than control subjects did [120]. And fourth, they showed that E-selectin-positive EEVs were associated with severe COPD and hyperinflation of the lungs (emphysema) [120]. Importantly, these results were found to be independent of other factors associated with endothelial injury, such as smoking status, sepsis, or the stage of the disease as classified by the Global Initiative on Obstructive Lung Disease. In addition, several studies demonstrated that the increased EEVs in COPD originated from the lung capillary endothelium [111], suggesting an initial regional dysfunction rather than a systemic process.

Although the main findings of these preclinical studies suggest that EEVs are a pathological trigger promoting endothelial injury in COPD, questions remain. Since COPD is an inflammatory lung disease in which multiple cells are involved, extracellular vesicles released by endothelial cells upon injury cannot be

viewed in isolation. As previously seen in ARDS, leukocytes, platelets, red blood cells, and epithelial cells also release extracellular vesicles with both beneficial and detrimental impacts on the endothelium, epithelium, coagulation, and immune cells. Further investigations are needed to shed new light on the complex array of extracellular vesicles involved in the pathogenesis of COPD, clarifying the potential use of EEVs as accurate biomarkers and as possible therapeutic targets.

### 38.7.3 Endogenous extracellular vesicles in asthma

Asthma is a common chronic inflammatory disease characterized by smooth muscle airway constriction and inflammation and reversible airflow obstruction. Multiple stimuli, such as allergens, infection, and air pollution, are potential triggers of exacerbations in asthma. Genetic factors also contribute to the severity of the symptoms. Immunopathology in asthma is characterized by a hyperresponsiveness of type 2 $CD4^+$ T helper cells which secrete proinflammatory cytokines such as IL-4, IL-5, and IL-13, which promote the influx of mast cells, type 2 $CD4^+$ T helper cells, and eosinophils. This specific shift toward a type 2 T helper proinflammatory response drives airway remodeling characterized by reversible airway hyperresponsiveness and airway obstruction [121–124]. As with other lung diseases, key cells involved in the pathology of asthma, such as mast cells, dendritic cells, T lymphocytes, and bronchial epithelial cells, are capable of releasing a broad spectrum of extracellular vesicles into their environment. Some of these vesicles promote the progression of the allergic reaction, whereas others exhibit a capacity for attenuating or blocking the allergic response.

Torregrosa Paredes *et al.* found that vesicles isolated from BAL fluid of asthmatic patients increased IL-8 and leukotriene C4 secretion in bronchial epithelial cells *in vitro*, suggesting a direct role of these vesicles in propagating the type 2 T helper cell proinflammatory response [125]. In addition, the authors speculated that the extracellular vesicles present in BAL fluid of asthmatic patients might originate mainly from the bronchial epithelium. This finding was in line with another study demonstrating that bronchial epithelial cells and lung macrophages were the main sources of vesicles in asthmatic patients. These investigators showed that IL-13-treated epithelial cells produced vesicles promoting macrophage expansion. Blocking vesicle generation protected mice from developing airway remodeling in a murine model of asthma [126]. In contrast, Prado *et al.* found that extracellular vesicles harvested from tolerized mice were capable of attenuating allergic sensitization, the immunoglobulin E response, and the type 2 T helper proinflammatory response, ultimately decreasing airway inflammation [127].

Unlike the situation in ARDS or COPD, where the biological effects of extracellular vesicles are mediated by multiple mechanisms, including proteins, receptors, mRNA, and microRNA, a growing body of preclinical evidence suggests that in asthma these effects are mainly driven by microRNA transfer from vesicles to the recipient cells. Multiple studies have found that microRNA was transferred from mast cells, dendritic cells, or T lymphocytes to other mast cells, dendritic cells, or antigen-presenting cells using extracellular vesicles as shuttle vectors [45,128,129]. Furthermore, there were significant differences in the microRNA content of exosomes involved in the IL-13 pathway. These included differences in members of the LETHAL-7 (LET-7) and miRNA-200 families in the BAL fluid of asthmatic patients compared with healthy controls [130]. Thus, overall endogenous extracellular vesicles appear to play an important role in asthma exacerbation, supporting the recent ongoing research using vesicles as biomarkers or as potential therapeutic targets.

### 38.7.4 Endogenous extracellular vesicles as biomarkers in lung disease

The composition of circulating extracellular vesicles in healthy humans can often be changed significantly by both physiological and pathological conditions. Normally, circulating extracellular vesicles are mainly derived from platelets and, to a lesser extent, from leukocytes and endothelial cells [131,132]. However, even physiological changes such as exercise, menstrual cycle, or age are capable of impacting the pattern of extracellular vesicle release in terms of quantity, size, heterogeneity, and phenotype [131,133,134]. Thus, extracellular vesicles have been studied as potential biomarkers in a wide variety of systemic inflammatory diseases, including atherosclerosis, vasculitis, pulmonary hypertension, cardiopulmonary resuscitation, systemic lupus erythematosus, Crohn's disease, rheumatoid arthritis, systemic inflammatory response syndrome, sepsis, and multi-organ dysfunction syndrome [90,132,135–142]. For example, based on the extensive preclinical studies of extracellular vesicles in ARDS pathophysiology, several groups have studied the potential use of extracellular vesicles as biomarker measured either in blood or BAL fluid from ARDS patients [13]. Since different proinflammatory stimuli (e.g., endotoxin, *N*-formylmethionyl-leucyl-phenylalanine, and phorbol myristate acetate) have increased the amount of extracellular vesicles released by diverse cell types, we can speculate that the amount of vesicles released into the blood and the alveolar space of ARDS patients would be higher. Although this field of research is still expanding, some studies have already found that a high circulating concentration of leukocyte extracellular vesicles was associated with better outcomes [81], whereas a high level of extracellular-vesicle-associated TF was associated with progression of the disease [13]. More studies investigating the possibility of exploiting extracellular vesicles as biomarkers in ARDS are expected soon. Likewise, higher circulating levels of vascular endothelial-cadherin, platelet endothelial cell adhesion molecule-1, and E-selectin-positive EEVs have been found in stable COPD patients than in non-COPD patients [118]. Furthermore, the same investigators suggested that E-selectin-positive EEV levels may be used as a biomarker of the severity of COPD, since they correlated with the acceleration of loss of lung function and the number of exacerbations [102,118]. Also, different levels of extracellular-vesicle-associated microRNA involved in the IL-13 pathway

measured in BAL fluid have been used to distinguish asthmatic patients from healthy controls [130], suggesting a potential use of extracellular vesicles as a biomarker in asthma.

However, as discussed earlier, extracellular vesicles are not inert particles released by cells upon specific stimuli, with minimal or no role in the progression of lung diseases. These vesicles also carry a large amount of bioactive molecules capable of impacting critical pathways involved in inflammation, coagulation, lung permeability, and endothelial function. Their multiple interactions with epithelial cells, endothelial cells, PMNs, monocytes, macrophages, lymphocytes, and platelets give rise to the possibility of modulating their functional effect as a therapeutic target for a number of lung diseases.

### 38.7.5 Endogenous extracellular vesicles as potential therapeutic targets for lung diseases

Because of their considerable heterogeneity in terms of origin and functional properties, extracellular vesicles may either stimulate or inhibit the characteristic pathological features of ARDS. Removal or inhibition of extracellular vesicles with pathological effects [143,144] and/or administration or stimulation of extracellular vesicles that attenuate multiple pathways involved in inflammatory lung diseases may give rise to new therapeutic strategies to modulate coagulation, inflammation, and endothelial injury with increased permeability.

One strategy demonstrated in a clinical study involved removal of significant amount of PEVs using a membrane plasma separator [145]. Although the method may be challenging and with an incomplete effect, advanced plasmapheresis technologies may enable a more selective separation allowing targeted removal of extracellular vesicles with pathological effects. Currently, pharmacological modulation of the deleterious extracellular vesicles may represent a more accessible goal. For example, glucocorticoids have been used to decrease the circulating level of PEVs in the nonpulmonary inflammatory diseases dermatomyositis and polymyositis [146]. Given the controversies around corticosteroid treatment and their frequent use as a therapeutic agent in many cases of persistent (>14 days) ARDS and COPD exacerbations, further investigation on the impact of corticosteroids on extracellular vesicles is needed. Furthermore, agonists of peroxisome proliferator-activated receptor-γ, such as rosiglitazone, have been found to inhibit important extracellular-vesicle-related inflammatory pathways involved in monocyte-associated extracellular vesicles and lymphocyte extracellular vesicles [90,147,148]. Although peroxisome proliferator-activated receptor-γ can attenuate experimental ARDS [149–151], the therapeutic effect on extracellular vesicle activity is still unknown. Finally, although statin and polyunsaturated fatty acids have been shown to modulate PEV formation [70,152], they were unsuccessful in preventing ARDS [153] or COPD exacerbations [154], suggesting that their impact on extracellular vesicle activity might be insufficient for contributing to a therapeutic effect.

As described in the previous sections, some of the endogenous released extracellular vesicles can exert beneficial effects on inflammation, coagulation, and endothelial function during lung injury. The concept of exploiting these capacities by administering anti-inflammatory, anticoagulant, and endothelial barrier-protective extracellular vesicles is appealing but still needs to be investigated. In addition, enhancement of their therapeutic effect by either upstream preconditioning of the parent cells prior to the release of the extracellular vesicles or by loading exogenous extracellular vesicles with bioactive molecules might also be part of their pharmacological engineering worth considering in the future.

Lastly, one specific endogenous cell type still needs to be addressed: the MSCs. Although their number is limited at baseline in multiple organs, including the lung, MSCs are capable of releasing a wide array of extracellular vesicles exhibiting largely an anti-inflammatory phenotype, which may be beneficial for lung diseases [155–158]. We will review in the next sections studies focused on the effect of MSC extracellular vesicle administration in organ injury, and more specifically in lung diseases.

## 38.8 Therapeutic properties of extracellular vesicles derived from mesenchymal stromal cells

In various organ injury models, MSC-derived vesicles, whether exosomes or MVs, have been shown to be as potent as the parent cell as a therapeutic agent in both tissue repair and/or regeneration (Figure 38.3).

The mechanisms have been primarily through the transfer of bioactive content from the vesicles to the recipient cells, changing the function and/or phenotype of the recipient cell. A systematic review [159] summarized the existing preclinical data with regard to the reparative and regenerative properties of MSC vesicles following injuries to the kidney, heart, liver, brain,and hind limb ischemia injury [160]. Based on such promising preclinical data, there is currently one phase 2/3 clinical trial using umbilical-cord-blood-derived MSC MVs in type 1 diabetes mellitus (NCT02138331).

### 38.8.1 Mesenchymal stromal cell vesicles for kidney injury

MSC-derived vesicles have been studied as a potential therapeutic agent in AKI models induced by cisplatin [161,162], glycerol [36], ischemia–reperfusion injury [163,164], nephrectomy [165], and drug toxicity (gentamicin) [166]. Overall, in comparisons with controls, MSC exosomes or MVs improved renal function and reduced the extent of kidney injury [18,167,168]. In an AKI model in severe combined immunodeficiency mice, Bruno *et al.* [36] found that MSC MV administration improved recovery from AKI in part by preventing apoptosis and increasing renal tubular epithelial cell proliferation. A single administration of MSC MVs immediately following cisplatin [161] or ischemia–

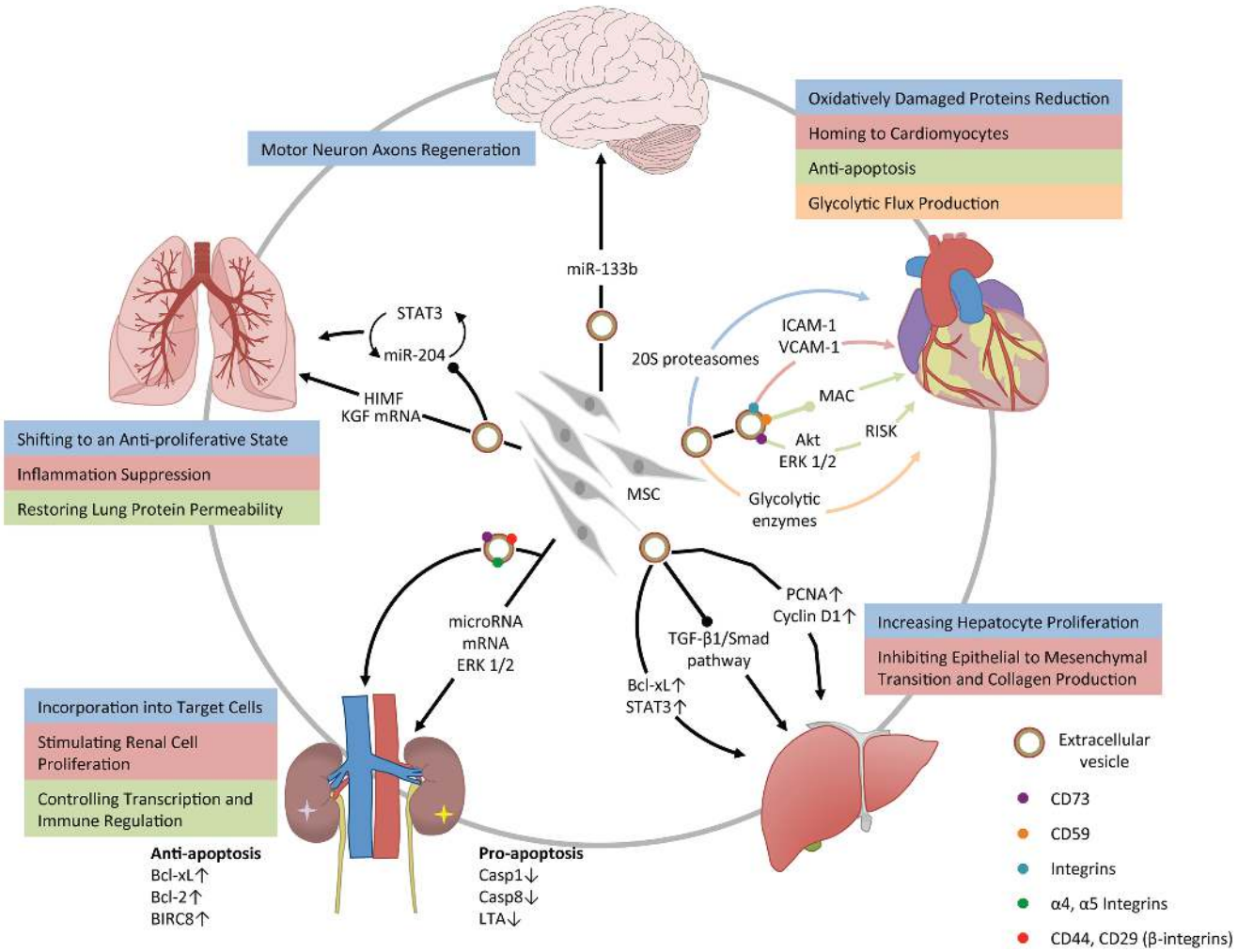



**Figure 38.3** Mechanisms underlying the therapeutic properties of extracellular vesicles derived from MSCs following different organ injuries. (See color plate section.)
*Kidney.* MSC-derived vesicles expressed several adhesion molecules, such as CD44, CD29 ($\beta_1$-integrin), $\alpha_4$ and $\alpha_5$ integrins, and CD73, which were critical for the incorporation into target cells. MSC MVs shuttled microRNA and mRNA into recipient cells and stimulated renal cell proliferation by activation of ERK1/2. In addition, MSC MVs conferred an antiapoptotic phenotype necessary for tissue repair by inducing the expression of anti-apoptotic genes (*BCL-XL*, *BCL2*, and *BIRC8*) in renal tubular epithelial cells while downregulating pro-apoptotic genes (*CASP1*, *CASP8*, and *LTA*).
*Heart.* MSC exosomes contained (1) integrins that could channel exosomes to cardiomyocytes that expressed ICAM-1, a ligand for integrins, or to VCAM-1 on endothelial cells; (2) CD73, present on the surface of exosomes, that activated RISK by increased expression of pro-survival protein kinases such as AKT and ERK1/2; (3) CD59 (protectin), a widely expressed glycosylphosphatidylinositol-anchored membrane protein that prevented the formation of MAC and inhibited complement-mediated lysis; (4) a significant clustering of enzymes involved in glycolysis, which could ameliorate a glycolytic deficit and potentially increase glycolytic flux and ATP production; additionally, they mediated the transfer of functionally active 20S proteasomes, which were responsible for the degradation of intracellular protein damaged by oxidation.
*Liver.* MSC exosomes inhibited epithelial-to-mesenchymal transition and collagen production by suppressing the activation of the TGF-β1/SMAD signaling pathway. MSC exosomes were also associated with higher expression of proliferation proteins (PCNA and cyclin D1), the antiapoptosis gene (*BCL-XL*), and higher expression of STAT3.
*Brain.* MSC exosomes transferred functional microRNAs, such as miR-133b, involved in regeneration of motor neuron axons to injured neural cells.
*Lung.* In acute lung injury, MSC exosomes suppressed inflammation by upregulating HIMF transcription and restored lung protein permeability by transferring KGF mRNA. In a model of PAH, MSC exosomes suppressed the hypoxic induction of STAT3 and upregulated miR-204 levels, interfering with the STAT3–miR-204–STAT3 feed-forward loop and shifting the balance to an antiproliferative state.
AKT: protein kinase B; BCL2: B-cell lymphoma 2; BCL-XL: B-cell lymphoma-extra large; BIRC8: baculoviral inhibitor of apoptosis repeat containing protein 8; Casp1: caspase 1; Casp8: caspase 8; CD: cluster of differentiation; ERK: extracellular regulated kinase; HIMF: hypoxia-induced mitogenic factor; ICAM-1: intercellular adhesion molecule-1; KGF: keratinocyte growth factor; LTA: lymphotoxin-α; MAC: membrane attack complex; MSC: mesenchymal stromal cell; PCNA: proliferating cell nuclear antigen; RISK: reperfusion injury salvage kinases; STAT3: signal transducer and activator of transcription 3; TGF-β1: transforming growth factor-β1; VCAM-1: vascular cell adhesion molecule 1.

reperfusion-injury-induced AKI [164] alleviated inflammation, mitigated renal cell apoptosis, and enhanced proliferation of the renal epithelial cells [164]. In addition, multiple injections of MSC MVs further improved renal function and morphology, abrogated renal fibrosis, and decreased mortality. When compared with MSCs, MVs were found to mimic the beneficial effects of the adult parent cells. The effects appeared to be mediated in part by the transfer of RNA by MSC MVs to the injured renal epithelium, as indicated by the loss of reparative effects after RNase pretreatment of the MVs [17,36,169]. Specifically, Bruno *et al.* demonstrated that MSC MVs shuttled RNA associated with control of transcription, proliferation, and immune regulation to the injured epithelium. The effective transfer of specific MV-derived microRNA and mRNA and the translation of MV-shuttled mRNA into proteins within recipient cells were shown both *in vitro* and *in vivo* [30,36]. In the AKI model, MSC MVs conferred an antiapoptotic phenotype necessary for tissue repair by inducing the expression of the antiapoptotic genes *BCL-XL*, *BCL2*, and *BIRC8* in renal tubular epithelial cells while simultaneously downregulating the pro-apoptotic genes *CASP1*, *CASP8*, and *LTA* [161]. In addition, MSC MVs stimulated renal cell proliferation by inducing the phosphorylation and subsequent activation of extracellular regulated kinase (ERK) 1/2 [162]. The authors found that MSC MVs expressed several adhesion molecules, such as CD44, CD29 ($\beta_1$-integrin), $\alpha_4$ and $\alpha_5$ integrins, and CD73, which were critical for the incorporation of the MVs into the target cells [36,169–171]. These data demonstrate the ability of MSC MVs to modulate simultaneously several different pathways to stimulate renal repair and/or regeneration.

### 38.8.2 Mesenchymal stromal cell vesicles for cardiac injury

The therapeutic effects of MSC vesicles have also been reported in several experimental models of myocardial ischemia–reperfusion injury [41,172–174]. For example, Timmers *et al.* previously found that conditioned medium from human embryonic stem-cell-derived MSCs significantly reduced infarct size in pig and mouse models of myocardial ischemia–reperfusion injury [175]. Using electron microscopy, ultracentrifugation, mass spectrometry, and biochemical assays the authors demonstrated that the active components of the conditioned medium were exosomes [172,176]. Administration of purified MSC exosomes 5 min before reperfusion significantly reduced infarct size and improved left ventricular function.

Several mechanisms for the therapeutic effect of MSC exosomes in myocardial ischemia–reperfusion injury were postulated.

First, exosomes contained membrane proteins that have significant binding affinity to other ligands on cell membranes or to the extracellular matrix. Specifically, integrins could channel exosomes to cardiomyocytes that expressed ICAM-1, a ligand for integrins, after myocardial ischemia–reperfusion injury [177] or to VCAM-1 on endothelial cells [178]. Tetraspanin proteins, which function primarily to mediate cellular penetration, invasion, and fusion events [179], could also facilitate cellular uptake of exosomes. Interestingly, it was observed that the efficiency of exosome uptake correlated directly with intracellular and microenvironmental acidity [180]. This may be a mechanism by which MSC exosomes exert their cardio-protective effects on ischemic cardiomyocytes that have a low intracellular pH [181].

Second, exosomes contain enzymes, and since enzyme activities are catalytic rather than stoichiometric and are dictated by their microenvironment (e.g., substrate concentration or pH), enzyme-based therapeutic activities of exosomes could be activated or attenuated according to the release of injury-associated substrates, which is directly proportional to the severity of the injured microenvironment [176]. Lai *et al.* interrogated the proteome of MSC exosomes by mass spectrometry and antibody array and found a significant clustering of enzymes involved in glycolysis. These enzymes from MSC exosomes could ameliorate the glycolytic deficit and potentially increase glycolytic flux and ATP production in the reperfused myocardium [42]. The intracellular transfer of glycolytic enzymes from MSC exosomes across the plasma membrane is likely to be sufficient for the enzymes to exert their biochemical activity, as glycolysis is a cytosolic process, unlike fatty acid oxidation and the tricarboxylic acid cycle, which are mitochondrial processes. An important corollary of this hypothesis is that MSC exosomes increased ATP production in reperfused myocardium and/or in cells where oxidative phosphorylation was inhibited [41,42]. However, Islam *et al.* found MSCs using connexin43 gap junctions were able to restore ATP levels in injured alveolar epithelium through the transfer of mitochondria in a mouse model of acute lung injury [43].

Third, activation of some pro-survival protein kinases such as AKT and ERK 1/2, which prevent apoptosis, has been demonstrated to be protective against myocardial ischemia–reperfusion injury when stimulated at the time of myocardial reperfusion [182,183]. These kinases are termed the reperfusion-injury salvage kinases (RISKs) [184]. From the proteomic study of MSC exosomes, Lai *et al.* also postulated that CD73, which was present on the surface of MSC exosomes, was the most likely protein candidate to overcome the pro-apoptotic proteome of reperfused myocardium through the activation of RISKs [42].

Fourth, MSC exosomes may possess additional therapeutic activities to prevent other complications of myocardial ischemia–reperfusion injury by reducing misfolded or oligomerized proteins in reperfused hearts through the transfer of functionally active 20S proteasomes, which are responsible for the degradation of ~90% of all intracellular protein damaged by oxidation [185]. In addition, CD59 (protectin), a widely expressed glycosylphosphatidylinositol-anchored membrane protein, is also present on MSC exosomes [186]. This principal molecule inhibits the terminal pathway of complement preventing the formation of the membrane attack complex (MAC). Thus, exosome-associated CD59 may contribute to cell protection by inhibition of complement-mediated lysis.

### 38.8.3 Mesenchymal stromal cell vesicles for liver injury

Two studies have focused on the therapeutic effects of MSC exosomes in a mouse model of liver injury. Using a carbon-tetrachloride-induced liver injury model [187,188], the investigators demonstrated that MSC exosomes ameliorated liver fibrosis by inhibiting the epithelial-to-mesenchymal transition of hepatocytes and collagen production. Exosomes were found to significantly restore the hepatic enzyme aspartate aminotransferase activity in serum and decrease collagen type I/III, TGF-β1 and phosphorylation of SMAD2 expression *in vivo*, suggesting the inactivation of TGF-β1/SMAD signaling pathway. In another study [188], the investigators found that MSC exosomes elicited hepatoprotective effects in acetaminophen and hydrogen-peroxide-induced liver injury primarily through an increase in hepatocyte proliferation, as demonstrated by elevation of the proliferating cell nuclear antigen (PCNA) and higher cell viability. The increased survival rate was associated with upregulation of the priming phase genes involved during liver regeneration, which subsequently led to higher expression of proliferation proteins (PCNA and cyclin D1) and the anti-apoptosis gene *BCL-XL* and higher expression of STAT3. Surprisingly, however, the therapeutic effect of MSC exosomes was not mediated through modulation of oxidative stress during hepatic injury.

### 38.8.4 Mesenchymal stromal cell vesicles for neural injury

The therapeutic role of MSC exosomes has been studied in a few models of neural ischemia. Yu *et al.* demonstrated that functional microRNAs, such as miR-133b, which is involved in regeneration of motor neuron axons [189], are transferred from MSCs to injured neural cells via exosomes, and Xin *et al.* found that exosome-encapsulated transfer of microRNAs promoted neurite remodeling and functional recovery following stroke in rats [190,191]. In a following study, the latter investigators administered cell-free MSC exosomes to rats subjected to middle cerebral artery occlusion and assessed functional recovery and enhancement of neurite remodeling, neurogenesis, and angiogenesis [192]. By evaluating functional recovery with a foot fault test [193] and a modified neurologic severity score [194] it was concluded that MSC exosomes contributed to significant functional improvement following middle cerebral artery occlusion. MSC exosome treatment also significantly increased axonal density and synaptophysin-positive areas along the ischemic boundary zone of the cortex and striatum, as well as the number of newly formed doublecortin$^+$ cells (neuroblasts) and von Willebrand factor$^+$ cells (endothelial cells).

Another group investigated the protective effects of rat bone marrow-derived MSC MVs against apoptosis in glutamate-induced injury in rat pheochromocytoma PC12 cells [195]. They demonstrated that MVs reversed both the glutamate-induced downregulation of AKT phosphorylation and Bcl-2 expression and the glutamate-induced upregulation of Bax expression and the level of cleaved caspase-3, suggesting that MVs act as cell-protective effects via the PI3K/AKT signaling pathway.

### 38.8.5 Mesenchymal stromal cell vesicles for lung diseases

To date, only a few groups have studied the effects of MSC vesicles in lung diseases such as pulmonary artery hypertension (PAH) [196,197] and acute lung injury [14]. Although the mechanisms of action have not been fully defined, these groups have demonstrated that MSC vesicles are as potent as their parent cells as therapy, suggesting a novel cell-derived preparation without the limitations of using live cells.

Lee *et al.* [196] demonstrated that exosomes mediated the cytoprotective effect of bone-marrow-derived MSCs in hypoxia-induced PAH in mice. In this model, administration of MSC-derived exosomes protected against the elevation of right ventricular systolic pressure and the development of right ventricular hypertrophy after 3 weeks of hypoxic exposure. Specifically, treatment with MSC exosomes interfered with the early hypoxic signal in the lung, suppressed inflammation, and upregulated HIMF transcription and alveolar macrophage activation. Surprisingly, exosome-depleted conditioned media had no therapeutic effect, suggesting a limited role for soluble factors released by MSCs. MSC exosomes also suppressed the hypoxic induction of STAT3 in primary cultures of pulmonary artery endothelial cells and upregulated miR-204 levels, interfering with the STAT3–miR-204–STAT3 feed-forward loop and shifting the balance to an antiproliferative state. Similarly, Chen *et al.* demonstrated that MSC MVs decreased pulmonary pressure and right ventricular pressure and reduced right ventricular hypertrophy and pulmonary arteriole remodeling during the development of PAH in rats [197].

Zhu *et al.* demonstrated a biologic effect of MVs derived from human bone marrow MSCs in a mouse model of endotoxin-induced acute lung injury [14]. Treatment with MSC MVs was effective in restoring lung protein permeability, reduced inflammation (such as an influx of neutrophils and elevation of macrophage inflammatory protein-2 levels), and prevented the formation of pulmonary edema in the injured alveoli. Moreover, KGF small interfering RNA pretreatment of MSCs partially eliminated the therapeutic effects of MSC MVs, suggesting that KGF protein expression was an important component of the underlying mechanism. Previously, KGF secretion by MSCs had been found to be involved in restoring both vectorial ion and fluid transport in injured human alveolar epithelial type II cells and in increasing the antimicrobial properties of monocytes and macrophages [6].

Although the few preclinical studies are promising, more work is needed to better understand the mechanisms underlying the therapeutic effects of MSC vesicles. For instance, it is unclear whether MSC vesicles retain the antimicrobial properties of MSCs or whether MSC vesicles can be used in other acute or chronic lung diseases, such as asthma and idiopathic pulmonary fibrosis, where the pathophysiology is different.

## 38.9 Remaining questions on the therapeutic use of mesenchymal stromal cell extracellular vesicles

The use of MSC-derived extracellular vesicles as a cell-free therapeutic agent in lung disease offers several advantages compared with using the parent cell from which the vesicles are derived. First, extracellular vesicles are non-self-replicating and can easily be stored at −80 °C for several months while maintaining their functional effect [198]. The risk of ectopic tissue formation or malignant transformation by MSC-derived extracellular vesicles is minimal. Second, extracellular vesicles in theory could be harvested from immortalized or prestimulated MSCs, which would drastically reduce the total number of MSCs needed. This approach could also lead to specific phenotypes of extracellular vesicles with enhanced therapeutic properties. Lastly, owing to their lower expression of membrane-bound proteins, extracellular vesicles may be less immunogenic than their parent cells [199]. However, multiple questions remain that must be addressed before effective translation to the clinic can take place.

### 38.9.1 Isolation and quantification techniques

Based on current preclinical studies on the therapeutic use of extracellular vesicles derived from MSCs, there are clear limitations using the current isolation and quantification techniques that require further research. First, the use of ultracentrifugation to isolate MSC vesicles does not differentiate them based on the size of the vesicles. Although both MVs and exosomes have therapeutic properties [161,196], further studies are needed to determine the contribution of each to the overall therapeutic effect. Second, as with MSCs themselves, it remains unclear whether there is a dose-dependent response based on the quantity of the extracellular vesicles released. For example, one of the functional effects of extracellular vesicles may be through direct stimulation of a target cell by a surface ligand on the extracellular vesicles. However, not all vesicles will have the same surface composition, despite being released from the same parent cell.

### 38.9.2 Extracellular vesicle characterization

The available techniques used for characterizing extracellular vesicles, such as proteomics, mass spectrometry, and microarrays, have evolved [13]; unfortunately, there is still no uniform standard for characterizing extracellular vesicles isolated by different investigators. More importantly, the different methods used to precondition MSCs in order to stimulate vesicle release, such as serum starvation, hypoxia, and inflammation, will change the surface and intracellular content of the released vesicles. Whether it will change the phenotype or effect of the released vesicles needs to be studied further. In addition, soluble factors released by MSCs appear to have therapeutic efficacy in a variety of inflammatory disorders, and released vesicles do contain these proteins [1–9]. However, the role of these proteins within the vesicles is unclear. In order to proceed toward translational application of extracellular vesicles in human lung disease, it is essential that optimal characterization methods be developed that will give rise to a consensus for standardizing the potency of these vesicles.

### 38.9.3 Feasibility of large-scale generation of extracellular vesicles

Based on the few studies using extracellular vesicles as treatment in preclinical animal models, roughly a 10-fold higher dose of extracellular vesicles was required to generate an effect equivalent to the parent cells [14,161,196]. Since the average therapeutic dose of MSCs administered systemically in the majority of ongoing clinical trials is between $1 \times 10^6$ and $2 \times 10^6$ MSCs/kg, there may be a need to harvest extracellular vesicles released in the region of $10–20 \times 10^6$ of MSCs/kg of body weight to get an equivalent effect. Consequently, large-scale generation of extracellular vesicles remains challenging. However, one published [200] and several ongoing clinical trials (NCT01159288, NCT01550523, NCT01779583 [201–203]) have demonstrated that this is feasible. Although several high-throughput techniques – such as centrifugation combined with either ultrafiltration or high-pressure liquid chromatography, or differential ultracentrifugation combined with a sucrose cushion, – have been proposed as viable manufacturing processes, large-scale extracellular vesicle production needs to be further developed, especially if prestimulation of the parent cells is used to generate a unique phenotype.

## 38.10 Regulatory considerations for the clinical use of extracellular vesicles

It is likely that extracellular vesicles will soon be considered as a biotherapy and, therefore, will need to comply with the rules and standards of regulatory authorities. Written protocols, standard operating procedures, quality control, and quality assurance methods have been in place for some time to govern the use of *ex vivo* expanded MSCs and other cellular therapies in clinical trials, including the need for appropriately licensed good manufacturing practice laboratory facilities (see Chapters 42–44). Similar demands will be placed on the clinical trial use of extracellular vesicles, which will need to demonstrate their safety for systemic distribution, purity, viability, sterility, potency, lack of toxicity, and half-life studies.

## 38.11 Conclusions

Although still in its infancy compared with MSC-based administration, there are potential advantages of using MSC-derived extracellular vesicles in a variety of inflammatory lung diseases, including asthma, ARDS, and COPD. However, questions remain concerning their characterization, potency, and quantification, making a direct comparison with MSCs difficult. More

research is needed on the mechanisms underlying the apparent beneficial effects of MSC-derived extracellular vesicles before making assumptions that extracellular vesicles are equivalent in potential efficacy to that of their parent stromal cells.

## References


1 N. Gupta, X. Su, B. Popov, *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;**179**:1855–1863.

2 J. Xu, J. Qu, L. Cao, *et al.* Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *J Pathol* 2008;**214**:472–481.

3 S.H. Mei, S.D. McCarter, Y. Deng, *et al.* Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 2007;**4**:e269.

4 N. Gupta, A. Krasnodembskaya, M. Kapetanaki, *et al.* Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia. *Thorax* 2012;**67**:533–539.

5 A. Krasnodembskaya, Y. Song, X. Fang, *et al.* Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010;**28**:2229–2238.

6 J.W. Lee, A. Krasnodembskaya, D.H. McKenna, *et al.* Therapeutic effects of human mesenchymal stem cells in ex vivo human lungs injured with live bacteria. *Am J Resp Crit Care Med* 2013;**187**:751–760.

7 K. Nemeth, A. Leelahavanichkul, P.S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

8 E. Gonzalez-Rey, P. Anderson, M.A. González, *et al.* Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009;**58**:929–939.

9 S.H. Mei, J.J. Haitsma, C.C. Dos Santos, *et al.* Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010;**182**:1047–1057.

10 R. Izadpanah, D. Kaushal, C. Kriedt, *et al.* Long-term *in vitro* expansion alters the biology of adult mesenchymal stem cells. *Cancer Res* 2008;**68**:4229–4238.

11 G.V. Rosland, A. Svendsen, A. Torsvik, *et al.* Long-term cultures of bone marrow-derived human mesenchymal stem cells frequently undergo spontaneous malignant transformation. *Cancer Res* 2009;**69**:5331–5339.

12 D.J. Prockop, M. Brenner, W.E. Fibbe, *et al.* Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;**12**:576–578.

13 M. McVey, A. Tabuchi, W.M. Kuebler. Microparticles and acute lung injury. *Am J Physiol Lung Cell Mol Physiol* 2012;**303**: L364–L381.

14 Y.G. Zhu, X.M. Feng, J. Abbott, *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;**32**:116–125.

15 K.W. Witwer, E.I. Buzás, L.T. Bemis, *et al.* Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. *J Extracell Vesicles* 2013;**2**. doi: 10.3402/jev.v2i0.20360.

16 S.J. Gould, G. Raposo. As we wait: coping with an imperfect nomenclature for extracellular vesicles. *J Extracell Vesicles* 2013;**2**. doi: 10.3402/jev.v2i0.20389.

17 L. Biancone, S. Bruno, M.C. Deregibus, *et al.* Therapeutic potential of mesenchymal stem cell-derived microvesicles. *Nephrol Dial Transplant* 2012;**27**:3037–3042.

18 A. Dorronsoro, P.D. Robbins. Regenerating the injured kidney with human umbilical cord mesenchymal stem cell-derived exosomes. *Stem Cell Res Ther* 2013;**4**:39.

19 B. György, T.G. Szabó, M. Pásztói, *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;**68**:2667–2688.

20 R.J. Simpson, S.S. Jensen, J.W. Lim. Proteomic profiling of exosomes: current perspectives. *Proteomics* 2008;**8**:4083–4099.

21 S. Mathivanan, H. Ji, R.J. Simpson. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* 2010;**73**:1907–1920.

22 C. Thery, M. Ostrowski, E. Segura. Membrane vesicles as conveyors of immune responses. *Nat Rev Immunol* 2009;**9**:581–593.

23 B. Zhang, Y. Yin, R.C. Lai, *et al.* Mesenchymal stem cells secrete immunologically active exosomes. *Stem Cells Dev* 2014;**23**:1233–1244.

24 S. Mathivanan, R.J. Simpson. ExoCarta: a compendium of exosomal proteins and RNA. *Proteomics* 2009;**9**:4997–5000.

25 H. Kalra, R.J. Simpson, H. Ji, *et al.* Vesiclepedia: a compendium for extracellular vesicles with continuous community annotation. *PLoS Biol* 2012;**10**:e1001450.

26 G. Camussi, M.C. Deregibus, S. Bruno, *et al.* Exosomes/microvesicles as a mechanism of cell-to-cell communication. *Kidney Int* 2010;**78**:838–848.

27 J. Ratajczak, K. Miekus, M. Kucia, *et al.* Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. *Leukemia* 2006;**20**:847–856.

28 M.C. Deregibus, V. Cantaluppi, R. Calogero, *et al.* Endothelial progenitor cell derived microvesicles activate an angiogenic program in endothelial cells by a horizontal transfer of mRNA. *Blood* 2007;**110**:2440–2448.

29 A. Yuan, E.L. Farber, A.L. Rapoport, *et al.* Transfer of microRNAs by embryonic stem cell microvesicles. *PloS One* 2009;**4**:e4722.

30 F. Collino, M.C. Deregibus, S. Bruno, *et al.* Microvesicles derived from adult human bone marrow and tissue specific mesenchymal stem cells shuttle selected pattern of miRNAs. *PloS One* 2010;**5**:e11803.

31 E. van der Pol, M.J. van Gemert, A. Sturk, *et al.* Single vs. swarm detection of microparticles and exosomes by flow cytometry. *J Thromb Haemost* 2012;**10**:919–930.

32 M. Mack, A. Kleinschmidt, H. Bruhl, *et al.* Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection. *Nat Med* 2000;**6**:769–775.

33 E.N. Nolte-'t Hoen, S.I. Buschow, S.M. Anderton, *et al.* Activated T cells recruit exosomes secreted by dendritic cells via LFA-1. *Blood* 2009;**113**:1977–1981.

34 H. Sheldon, E. Heikamp, H. Turley, *et al.* New mechanism for Notch signaling to endothelium at a distance by Delta-like 4 incorporation into exosomes. *Blood* 2010;**116**:2385–2394.

35 S.F. Mause, C. Weber. Microparticles: protagonists of a novel communication network for intercellular information exchange. *Circ Res* 2010;**107**:1047–1057.

36 S. Bruno, C. Grange, M.C. Deregibus, *et al.* Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;**20**:1053–1067.

37 G. Taraboletti, S. D'Ascenzo, I. Giusti, *et al.* Bioavailability of VEGF in tumor-shed vesicles depends on vesicle burst induced by acidic pH. *Neoplasia* 2006;**8**:96–103.

38 G. Taraboletti, S. D'Ascenzo, P. Borsotti, *et al.* Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Path* 2002;**160**:673–680.

39 S.S. Sidhu, A.T. Mengistab, A.N. Tauscher, *et al.* The microvesicle as a vehicle for EMMPRIN in tumor–stromal interactions. *Oncogene* 2004;**23**:956–963.

40 X. Li, F. Arslan, Y. Ren, *et al.* Metabolic adaptation to a disruption in oxygen supply during myocardial ischemia and reperfusion is underpinned by temporal and quantitative changes in the cardiac proteome. *J Proteome Res* 2012;**11**:2331–2346.

41 F. Arslan, R.C. Lai, M.B. Smeets, *et al.* Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem Cell Res* 2013;**10**:301–12.

42 R.C. Lai, R.W. Yeo, K.H. Tan, S.K. Lim. Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen Med* 2013;**8**:197–209.

43 M.N. Islam, S.R. Das, M.T. Emin, *et al.* Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 2012;**18**:759–765.

44 A. Sarkar, S. Mitra, S. Mehta, *et al.* Monocyte derived microvesicles deliver a cell death message via encapsulated caspase-1. *PloS One* 2009;**4**:e7140.

45 H. Valadi, K. Ekström, A. Bossios, *et al.* Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol* 2007;**9**:654–659.

46 M. Simons, G. Raposo. Exosomes – vesicular carriers for intercellular communication. *Curr Opin Cell Biol* 2009;**21**:575–581.

47 P.J. Quesenberry, J.M. Aliotta. The paradoxical dynamism of marrow stem cells: considerations of stem cells, niches, and microvesicles. *Stem Cell Rev* 2008;**4**:137–147.

48 L. Li, T. Xie. Stem cell niche: structure and function. *Annu Rev Cell Dev Biol* 2005;**21**:605–631.

49 N.T. Eissa. The exosome in lung diseases: message in a bottle. *J Allergy Clin Immunol* 2013;**131**:904–905.

50 B. Thebaud, D.J. Stewart. Exosomes: cell garbage can, therapeutic carrier, or Trojan horse? *Circulation* 2012;**126**:2553–2555.

51 V.M. Ranieri, G.D. Rubenfeld, B.T. Thompson, *et al.* Acute respiratory distress syndrome: the Berlin Definition. *JAMA* 2012;**307**:2526–2533.

52 L.B. Ware, M.A. Matthay. The acute respiratory distress syndrome. *N Engl J Med* 2000;**342**:1334–1349.

53 G.T. Brown, T.M. McIntyre. Lipopolysaccharide signaling without a nucleus: kinase cascades stimulate platelet shedding of proinflammatory IL-1β-rich microparticles. *J Immunol* 2011;**186**:5489–5496.

54 W. Jy, W.W. Mao, L. Horstman, *et al.* Platelet microparticles bind, activate and aggregate neutrophils *in vitro*. *Blood Cells Mol Dis* 1995;**21**:217–231.

55 S.C. Lo, C.Y. Hung, D.T. Lin, *et al.* Involvement of platelet glycoprotein Ib in platelet microparticle mediated neutrophil activation. *J Biomed Sci* 2006;**13**:787–796.

56 O.P. Barry, D. Pratico, J.A. Lawson, G.A. FitzGerald. Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *J Clin Invest* 1997;**99**:2118–2127.

57 G.B. Machado, M.C. de Assis, R. Leão, *et al.* ExoU-induced vascular hyperpermeability and platelet activation in the course of experimental *Pseudomonas aeruginosa* pneumosepsis. *Shock* 2010;**33**:315–321.

58 O.P. Barry, G.A. FitzGerald. Mechanisms of cellular activation by platelet microparticles. *Thromb Haemost* 1999;**82**:794–800.

59 O.P. Barry, M.G. Kazanietz, D. Pratico, G.A. FitzGerald. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. *J Biol Chem* 1999;**274**:7545–7556.

60 O.P. Barry, D. Pratico, R.C. Savani, G.A. FitzGerald. Modulation of monocyte–endothelial cell interactions by platelet microparticles. *J Clin Invest* 1998;**102**:136–144.

61 A. Angelillo-Scherrer. Leukocyte-derived microparticles in vascular homeostasis. *Circ Res* 2012;**110**:356–369.

62 M. Mesri, D.C. Altieri. Endothelial cell activation by leukocyte microparticles. *J Immunol* 1998;**161**:4382–4387.

63 M. Mesri, D.C. Altieri. Leukocyte microparticles stimulate endothelial cell cytokine release and tissue factor induction in a JNK1 signaling pathway. *J Biol Chem* 1999;**274**:23111–23118.

64 L.B. Ware. Pathophysiology of acute lung injury and the acute respiratory distress syndrome. *Semin Respir Crit Care Med* 2006;**27**:337–349.

65 O. Aras, A. Shet, R.R. Bach, *et al.* Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood* 2004;**103**:4545–4553.

66 T. van der Poll. Tissue factor as an initiator of coagulation and inflammation in the lung. *Crit Care* 2008;**12**:S3.

67 O. Morel, L. Jesel, J.M. Freyssinet, F. Toti. Cellular mechanisms underlying the formation of circulating microparticles. *Arterioscler Thromb Vasc Biol* 2011;**31**:15–26.

68 E.I. Sinauridze, D.A. Kireev, N.Y. Popenko, *et al.* Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost* 2007;**97**:425–434.

69 A. Aharon, T. Tamari, B. Brenner. Monocyte-derived microparticles and exosomes induce procoagulant and apoptotic effects on endothelial cells. *Thromb Haemost* 2008;**100**:878–885.

70 D. Lechner, A. Weltermann. Circulating tissue factor-exposing microparticles. *Thromb Res* 2008;**122**:S47–S54.

71 T. Benameur, R. Andriantsitohaina, M.C. Martinez. Therapeutic potential of plasma membrane-derived microparticles. *Pharmacol Rep* 2009;**61**:49–57.

72 W. Jy, M. Ricci, S. Shariatmadar, *et al.* Microparticles in stored red blood cells as potential mediators of transfusion complications. *Transfusion* 2011;**51**:886–893.

73 J. Rosing, H. Speijer, R.F. Zwaal. Prothrombin activation on phospholipid membranes with positive electrostatic potential. *Biochemistry* 1988;**27**:8–11.

74 J.A. Bastarache, R.D. Fremont, J.A. Kropski, *et al.* Procoagulant alveolar microparticles in the lungs of patients with acute respiratory distress syndrome. *Am J Physiol Lung Cell Mol Physiol* 2009;**297**:L1035–L1041.

75 J.A. Bastarache, S.C. Sebag, B.S. Grove, L.B. Ware. Interferon-γ and tumor necrosis factor-α act synergistically to up-regulate tissue factor in alveolar epithelial cells. *Exp Lung Res* 2011;**37**:509–517.

76 Y. Ishitsuka, H. Moriuchi, Y. Isohama, *et al.* A selective thromboxane A2 (TXA2) synthase inhibitor, ozagrel, attenuates lung injury and decreases monocyte chemoattractant protein-1 and interleukin-8 mRNA expression in oleic acid-induced lung injury in guinea pigs. *J Pharmacol Sci* 2009;**111**:211–215.

77 A. Zarbock, K. Singbartl, K. Ley. Complete reversal of acid-induced acute lung injury by blocking of platelet-neutrophil aggregation. *J Clin Invest* 2006;**116**:3211–3219.

78 K.L. Buesing, J.C. Densmore, S. Kaul, *et al.* Endothelial microparticles induce inflammation in acute lung injury. *J Surg Res* 2011;**166**:32–39.

79 J.C. Densmore, P.R. Signorino, J. Ou, *et al.* Endothelium-derived microparticles induce endothelial dysfunction and acute lung injury. *Shock* 2006;**26**:464–471.

80 E. Letsiou, S. Sammani, W. Zhang, *et al.* Pathologic mechanical stress and endotoxin exposure increases lung endothelial microparticle shedding. *Am J Resp Cell Mol Biol* 2015;**52**:193–204.

81 C. Guervilly, R. Lacroix, J.M. Forel, *et al.* High levels of circulating leukocyte microparticles are associated with better outcome in acute respiratory distress syndrome. *Crit Care* 2011;**15**:R31.

82 A.O. Soriano, W. Jy, J.A. Chirinos, *et al.* Levels of endothelial and platelet microparticles and their interactions with leukocytes negatively correlate with organ dysfunction and predict mortality in severe sepsis. *Crit Care Med* 2005;**33**:2540–2546.

83 K. Tang, J. Liu, Z. Yang, *et al.* Microparticles mediate enzyme transfer from platelets to mast cells: a new pathway for lipoxin A4 biosynthesis. *Biochem Biophys Res Commun* 2010;**400**:432–436.

84 F. Dignat-George, C.M. Boulanger. The many faces of endothelial microparticles. *Arterioscler Thromb Vasc Biol* 2011;**31**:27–33.

85 D. El Kebír, L. József, W. Pan, *et al.* 15-Epi-lipoxin A4 inhibits myeloperoxidase signaling and enhances resolution of acute lung injury. *Am J Resp Crit Care Med* 2009;**180**:311–319.

86 J. Dalli, L.V. Norling, D. Renshaw, *et al.* Annexin 1 mediates the rapid anti-inflammatory effects of neutrophil derived microparticles. *Blood* 2008;**112**:2512–2519.

87 O. Gasser, J.A. Schifferli. Activated polymorphonuclear neutrophils disseminate anti-inflammatory microparticles by ectocytosis. *Blood* 2004;**104**:2543–2548.

88 C.I. Timár, A.M. Lorincz, R. Csépányi-Kömi, *et al.* Antibacterial effect of microvesicles released from human neutrophilic granulocytes. *Blood* 2013;**121**:510–518.

89 N.C. Kaneider, A.J. Leger, A. Agarwal, *et al.* "Role reversal" for the receptor PAR1 in sepsis induced vascular damage. *Nat Immunol* 2007;**8**:1303–1312.

90 O. Morel, F. Toti, N. Morel, J.M. Freyssinet. Microparticles in endothelial cell and vascular homeostasis: are they really noxious? *Haematologica* 2009;**94**:313–317.

91 A. Agouni, H.A. Mostefai, C. Porro, *et al.* Sonic hedgehog carried by microparticles corrects endothelial injury through nitric oxide release. *FASEB J* 2007;**21**:2735–2741.

92 F. Meziani, A. Tesse, R. Andriantsitohaina. Microparticles are vectors of paradoxical information in vascular cells including the endothelium: role in health and diseases. *Pharmacol Rep* 2008;**60**:75–84.

93 H.A. Mostefai, A. Agouni, N. Carusio, *et al.* Phosphatidylinositol 3-kinase and xanthine oxidase regulate nitric oxide and reactive oxygen species productions by apoptotic lymphocyte microparticles in endothelial cells. *J Immunol* 2008;**180**:5028–5035.

94 K.F. Rabe, S. Hurd, A. Anzueto, *et al.* Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease: GOLD executive summary. *Am J Resp Crit Care Med* 2007;**176**:532–555.

95 P.M. Henson, R.W. Vandivier, I.S. Douglas. Cell death, remodeling, and repair in chronic obstructive pulmonary disease? *Proc Am Thorac Soc* 2006;**3**:713–717.

96 S. Hodge, G. Hodge, M. Holmes, P.N. Reynolds. Increased airway epithelial and T-cell apoptosis in COPD remains despite smoking cessation. *Eur Resp J* 2005;**25**:447–454.

97 Y. Kasahara, R.M. Tuder, C.D. Cool, *et al.* Endothelial cell death and decreased expression of vascular endothelial growth factor and vascular endothelial growth factor receptor 2 in emphysema. *Am J Resp Crit Care Med* 2001;**163**:737–744.

98 Y. Kasahara, R.M. Tuder, L. Taraseviciene-Stewart, *et al.* Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 2000;**106**:1311–1619.

99 S.W. Ryter, A.M. Choi. Autophagy in the lung. *Proc Am Thorac Soc* 2010;**7**:13–21.

100 T. Tsuji, K. Aoshiba, A. Nagai. Alveolar cell senescence in patients with pulmonary emphysema. *Am J Resp Crit Care Med* 2006;**174**:886–893.

101 R.W. Vandivier, P.M. Henson, I.S. Douglas. Burying the dead: the impact of failed apoptotic cell removal (efferocytosis) on chronic inflammatory lung disease. *Chest* 2006;**129**:1673–1682.

102 T. Takahashi, H. Kubo. The role of microparticles in chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis* 2014;**9**:303–314.

103 D. Conway, M.A. Schwartz. Lessons from the endothelial junctional mechanosensory complex. *F1000 Biol Rep* 2012;**4**:1.

104 M. Cicmil, J.M. Thomas, M. Leduc, *et al.* Platelet endothelial cell adhesion molecule-1 signaling inhibits the activation of human platelets. *Blood* 2002;**99**:137–144.

105 S. Falati, S. Patil, P.L. Gross, *et al.* Platelet PECAM-1 inhibits thrombus formation *in vivo*. *Blood* 2006;**107**:535–541.

106 K. Ley, C. Laudanna, M.I. Cybulsky, S. Nourshargh. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007;**7**:678–689.

107 W.A. Muller. Leukocyte–endothelial-cell interactions in leukocyte transmigration and the inflammatory response. *Trends Immunol* 2003;**24**:327–334.

108 S. Patil, D.K. Newman, P.J. Newman. Platelet endothelial cell adhesion molecule-1 serves as an inhibitory receptor that modulates platelet responses to collagen. *Blood* 2001;**97**:1727–1732.

109 Z. Wang, X. Yan. CD146, a multi-functional molecule beyond adhesion. *Cancer Lett* 2013;**330**:150–162.

110 A. Woodfin, M.B. Voisin, S. Nourshargh. PECAM-1: a multi-functional molecule in inflammation and vascular biology. *Arterioscler Thromb Vasc Biol* 2007;**27**:2514–2523.

111 C. Gordon, K. Gudi, A. Krause, *et al.* Circulating endothelial microparticles as a measure of early lung destruction in cigarette smokers. *Am J Resp Crit Care Med* 2011;**184**:224–232.

112 C. Heiss, N. Amabile, A.C. Lee, *et al.* Brief secondhand smoke exposure depresses endothelial progenitor cells activity and endothelial function: sustained vascular injury and blunted nitric oxide production. *J Am Coll Cardiol* 2008;**51**:1760–1771.

113 T. Takahashi, S. Kobayashi, N. Fujino, *et al.* Differences in the released endothelial microparticle subtypes between human

pulmonary microvascular endothelial cells and aortic endothelial cells *in vitro*. *Exp Lung Res* 2013;**39**:155–161.
114 S. Ge, B. Hertel, S.H. Emden, *et al.* Microparticle generation and leucocyte death in Shiga toxin-mediated HUS. *Nephrol Dial Transplant* 2012;**27**:2768–2775.
115 M. Kornek, M. Lynch, S.H. Mehta, *et al.* Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steato-hepatitis. *Gastroenterology* 2012;**143**:448–458.
116 E. Mayne, N.T. Funderburg, S.F. Sieg, *et al.* Increased platelet and microparticle activation in HIV infection: upregulation of P-selectin and tissue factor expression. *J Acquir Immune Defic Syndr* 2012;**59**:340–346.
117 R. Nieuwland, R.J. Berckmans, S. McGregor, *et al.* Cellular origin and procoagulant properties of microparticles in meningococcal sepsis. *Blood* 2000;**95**:930–935.
118 T. Takahashi, S. Kobayashi, N. Fujino, *et al.* Increased circulating endothelial microparticles in COPD patients: a potential biomarker for COPD exacerbation susceptibility. *Thorax* 2012;**67**:1067–1074.
119 T. Takahashi, S. Suzuki, H. Kubo, *et al.* Impaired endothelial progenitor cell mobilization and colony-forming capacity in chronic obstructive pulmonary disease. *Respirology* 2011;**16**:680–687.
120 M.A. Thomashow, D. Shimbo, M.A. Parikh, *et al.* Endothelial microparticles in mild chronic obstructive pulmonary disease and emphysema. The Multi-Ethnic Study of Atherosclerosis Chronic Obstructive Pulmonary Disease study. *Am J Respir Crit Care Med* 2013;**188**:60–68.
121 R.M. Locksley. Asthma and allergic inflammation. *Cell* 2010;**140**:777–783.
122 J. Lotvall, C.A. Akdis, L.B. Bacharier, *et al.* Asthma endotypes: a new approach to classification of disease entities within the asthma syndrome. *J Allergy Clin Immunol* 2011;**127**:355–360.
123 F.D. Martinez, D. Vercelli. Asthma. *Lancet* 2013;**382**:1360–1372.
124 R.M. Seguin, N. Ferrari. Emerging oligonucleotide therapies for asthma and chronic obstructive pulmonary disease. *Expert Opin Invest Drugs* 2009;**18**:1505–1517.
125 P. Torregrosa Paredes, J. Esser, C. Admyre, *et al.* Bronchoalveolar lavage fluid exosomes contribute to cytokine and leukotriene production in allergic asthma. *Allergy* 2012;**67**:911–919.
126 A. Kulshreshtha, T. Ahmad, A. Agrawal, B. Ghosh. Proinflammatory role of epithelial cell-derived exosomes in allergic airway inflammation. *J Allergy Clin Immunol* 2013;**131**:1194–1203.
127 N. Prado, E.G. Marazuela, E. Segura, *et al.* Exosomes from bronchoalveolar fluid of tolerized mice prevent allergic reaction. *J Immunol* 2008;**181**:1519–1525.
128 M. Mittelbrunn, C. Gutiérrez-Vázquez, C. Villarroya-Beltri, *et al.* Unidirectional transfer of microRNA-loaded exosomes from T cells to antigen-presenting cells. *Nat Commun* 2011;**2**:282.
129 A. Montecalvo, A.T. Larregina, W.J. Shufesky, *et al.* Mechanism of transfer of functional microRNAs between mouse dendritic cells via exosomes. *Blood* 2012;**119**:756–766.
130 B. Levanen, N.R. Bhakta, P. Torregrosa Paredes, *et al.* Altered microRNA profiles in bronchoalveolar lavage fluid exosomes in asthmatic patients. *J Allergy Clin Immunol* 2013;**131**:894–903.
131 V. Chaar, M. Romana, J. Tripette, *et al.* Effect of strenuous physical exercise on circulating cell-derived microparticles. *Clin Hemorheol Microcirc* 2011;**47**:15–25.
132 G.N. Chironi, C.M. Boulanger, A. Simon, *et al.* Endothelial microparticles in diseases. *Cell Tissue Res* 2009;**335**:143–151.
133 A. Forest, E. Pautas, P. Ray, *et al.* Circulating microparticles and procoagulant activity in elderly patients. *J Gerontol A Biol Sci Med Sci* 2010;**65**:414–420.
134 B. Toth, K. Nikolajek, A. Rank, *et al.* Gender-specific and menstrual cycle dependent differences in circulating microparticles. *Platelets* 2007;**18**:515–521.
135 N. Amabile, P.E. Rautou, A. Tedgui, C.M. Boulanger. Microparticles: key protagonists in cardiovascular disorders. *Semin Thromb Hemost* 2010;**36**:907–916.
136 C.M. Boulanger. Microparticles, vascular function and hypertension. *Curr Opin Nephrol Hypertens* 2010;**19**:177–180.
137 E.S. Breimo, B. Østerud. Generation of tissue factor-rich microparticles in an *ex vivo* whole blood model. *Blood Coagul Fibrinolysis* 2005;**16**:399–405.
138 K. Fink, L. Feldbrugge, M. Schwarz, *et al.* Circulating annexin V positive microparticles in patients after successful cardiopulmonary resuscitation. *Crit Care* 2011;**15**:R251.
139 A.P. Owens III, N. Mackman. Microparticles in hemostasis and thrombosis. *Circ Res* 2011;**108**:1284–1297.
140 M. Pirro, G. Schillaci, F. Bagaglia, *et al.* Microparticles derived from endothelial progenitor cells in patients at different cardiovascular risk. *Atherosclerosis* 2008;**197**:757–767.
141 P. Puddu, G.M. Puddu, E. Cravero, *et al.* The involvement of circulating microparticles in inflammation, coagulation and cardiovascular diseases. *Can J Cardiol* 2010;**26**:140–145.
142 J. Simak, M.P. Gelderman. Cell membrane microparticles in blood and blood products: potentially pathogenic agents and diagnostic markers. *Transfus Med Rev* 2006;**20**:1–26.
143 C. Camaioni, M. Gustapane, P. Cialdella, *et al.* Microparticles and microRNAs: new players in the complex field of coagulation. *Intern Emerg Med* 2013;**8**:291–296.
144 M. Kornek, Y. Popov, T.A. Libermann, *et al.* Human T cell microparticles circulate in blood of hepatitis patients and induce fibrolytic activation of hepatic stellate cells. *Hepatology* 2011;**53**:230–242.
145 N. Hanafusa, H. Satonaka, K. Doi, *et al.* Platelet-derived microparticles are removed by a membrane plasma separator. *ASAIO J* 2010;**56**:323–325.
146 T. Shirafuji, H. Hamaguchi, M. Higuchi, F. Kanda. Measurement of platelet-derived microparticle levels using an enzyme-linked immunosorbent assay in polymyositis and dermatomyositis patients. *Muscle Nerve* 2009;**39**:586–590.
147 G. Al-Massarani, H. Vacher-Coponat, P. Paul, *et al.* Kidney transplantation decreases the level and procoagulant activity of circulating microparticles. *Am J Transplant* 2009;**9**:550–557.
148 B. Köppler, C. Cohen, D. Schlöndorff, M. Mack. Differential mechanisms of microparticle transfer to B cells and monocytes: anti-inflammatory properties of microparticles. *Eur J Immunol* 2006;**36**:648–660.
149 Y. Aoki, T. Maeno, K. Aoyagi, *et al.* Pioglitazone, a peroxisome proliferator-activated receptor gamma ligand, suppresses bleomycin-induced acute lung injury and fibrosis. *Respiration* 2009;**77**:311–319.
150 D. Liu, B.X. Zeng, S.H. Zhang, *et al.* Rosiglitazone, a peroxisome proliferator-activated receptor-gamma agonist, reduces acute lung injury in endotoxemic rats. *Crit Care Med* 2005;**33**:2309–2316.

151 M.B. Schaefer, A. Pose, J. Ott, *et al.* Peroxisome proliferator-activated receptor-alpha reduces inflammation and vascular leakage in a murine model of acute lung injury. *Eur Resp J* 2008;**32**:1344–1353.

152 P. Davizon, A.D. Munday, J.A. López. Tissue factor, lipid rafts, and microparticles. *Semin Thromb Hemost* 2010;**36**:857–864.

153 J.D. Truwit, G.R. Bernard, J. Steingrub, *et al.* Rosuvastatin for sepsis-associated acute respiratory distress syndrome. *N Engl J Med* 2014;**370**:2191–2200.

154 G.J. Criner, J.E. Connett, S.D. Aaron, *et al.* Simvastatin for the prevention of exacerbations in moderate-to-severe COPD. *N Engl J Med* 2014;**370**:2201–2210.

155 J.E. Gotts, M.A. Matthay. Mesenchymal stem cells and acute lung injury. *Crit Care Clinics* 2011;**27**:719–733.

156 J.W. Lee, X. Fang, A. Krasnodembskaya, *et al.* Concise review: mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. *Stem Cells* 2011;**29**:913–919.

157 J.W. Lee, N. Gupta, V. Serikov, M.A. Matthay. Potential application of mesenchymal stem cells in acute lung injury. *Expert Opin Biol Ther* 2009;**9**:1259–1270.

158 M.A. Matthay, A. Goolaerts, J.P. Howard, J.W. Lee. Mesenchymal stem cells for acute lung injury: preclinical evidence. *Crit Care Med* 2010;**38**:S569–S573.

159 C. Akyurekli, Y. Le, R.B. Richardson, *et al.* A systematic review of preclinical studies on the therapeutic potential of mesenchymal stromal cell-derived microvesicles. *Stem Cell Rev* 2015;**11**:150–160.

160 H.C. Zhang, X.B. Liu, S. Huang, *et al.* Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev* 2012;**21**:3289–3297.

161 S. Bruno, C. Grange, F. Collino, *et al.* Microvesicles derived from mesenchymal stem cells enhance survival in a lethal model of acute kidney injury. *PloS One* 2012;**7**:e33115.

162 Y. Zhou, H. Xu, W. Xu, *et al.* Exosomes released by human umbilical cord mesenchymal stem cells protect against cisplatin-induced renal oxidative stress and apoptosis *in vivo* and *in vitro*. *Stem Cell Res Ther* 2013;**4**:34.

163 S. Gatti, S. Bruno, M.C. Deregibus, *et al.* Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia–reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant* 2011;**26**:1474–1483.

164 X. Zou, G. Zhang, Z. Cheng, *et al.* Microvesicles derived from human Wharton's jelly mesenchymal stromal cells ameliorate renal ischemia-reperfusion injury in rats by suppressing CX3CL1. *Stem Cell Res Ther* 2014;**5**:40.

165 J. He, Y. Wang, S. Sun, *et al.* Bone marrow stem cells-derived microvesicles protect against renal injury in the mouse remnant kidney model. *Nephrology* 2012;**17**:493–500.

166 L.A. Reis, F.T. Borges, M.J. Simoes, *et al.* Bone marrow-derived mesenchymal stem cells repaired but did not prevent gentamicin-induced acute kidney injury through paracrine effects in rats. *PloS One* 2012;**7**:e44092.

167 J.V. Bonventre. Microvesicles from mesenchymal stromal cells protect against acute kidney injury. *J Am Soc Nephrol* 2009;**20**:927–928.

168 M. Morigi, A. Benigni. Mesenchymal stem cells and kidney repair. *Nephrol Dial Transplant* 2013;**28**:788–793.

169 K. Sabin, N. Kikyo. Microvesicles as mediators of tissue regeneration. *Transl Res* 2014;**163**:286–295.

170 D.M. Iglesias, R. El-Kares, A. Taranta, *et al.* Stem cell microvesicles transfer cystinosin to human cystinotic cells and reduce cystine accumulation *in vitro*. *PloS One* 2012;**7**:e42840.

171 S. Bruno, G. Camussi. Role of mesenchymal stem cell-derived microvesicles in tissue repair. *Pediatr Nephrol* 2013;**28**:2249–2254.

172 R.C. Lai, F. Arslan, M.M. Lee, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214–222.

173 R.C. Lai, F. Arslan, S.S. Tan, *et al.* Derivation and characterization of human fetal MSCs: an alternative cell source for large-scale production of cardioprotective microparticles. *J Mol Cell Cardiol* 2010;**48**:1215–1224.

174 T.S. Chen, R.C. Lai, M.M. Lee, *et al.* Mesenchymal stem cell secretes microparticles enriched in pre-microRNAs. *Nucleic Acids Res* 2010;**38**:215–224.

175 L. Timmers, S.K. Lim, F. Arslan, *et al.* Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;**1**:129–137.

176 R.C. Lai, T.S. Chen, S.K. Lim. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* 2011;**6**:481–492.

177 G.L. Kukielka, H.K. Hawkins, L. Michael, *et al.* Regulation of intercellular adhesion molecule-1 (ICAM-1) in ischemic and reperfused canine myocardium. *J Clin Invest* 1993;**92**:1504–1516.

178 S. Rieu, C. Géminard, H. Rabesandratana, *et al.* Exosomes released during reticulocyte maturation bind to fibronectin via integrin $\alpha_4\beta_1$. *Eur J Biochem* 2000;**267**:583–590.

179 M.E. Hemler. Tetraspanin proteins mediate cellular penetration, invasion, and fusion events and define a novel type of membrane microdomain. *Ann Rev Cell Dev Biol* 2003;**19**:397–422.

180 I. Parolini, C. Federici, C. Raggi, *et al.* Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem* 2009;**284**:34211–34222.

181 J. Schrader. Mechanisms of ischemic injury in the heart. *Basic Res Cardiol* 1985;**80**:135–139.

182 D.J. Hausenloy, D.M. Yellon. New directions for protecting the heart against ischaemia–reperfusion injury: targeting the reperfusion injury salvage kinase (RISK)-pathway. *Cardiovasc Res* 2004;**61**:448–460.

183 D.M. Yellon, G.F. Baxter. Reperfusion injury revisited: is there a role for growth factor signaling in limiting lethal reperfusion injury? *Trends Cardiovasc Med* 1999;**9**:245–249.

184 D.J. Hausenloy, D.M. Yellon. Reperfusion injury salvage kinase signalling: taking a RISK for cardioprotection. *Heart Fail Rev* 2007;**12**:217–234.

185 R.C. Lai, S.S. Tan, B.J. Teh, *et al.* Proteolytic potential of the MSC exosome proteome: implications for an exosome-mediated delivery of therapeutic proteasome. *Int J Proteomics* 2012;**2012**:971907.

186 A. Clayton, C.L. Harris, J. Court, *et al.* Antigen presenting cell exosomes are protected from complement-mediated lysis by expression of CD55 and CD59. *Eur J Immunol* 2003;**33**:522–531.

187 T. Li, Y. Yan, B. Wang, *et al.* Exosomes derived from human umbilical cord mesenchymal stem cells alleviate liver fibrosis. *Stem Cells Dev* 2013;**22**:845–854.

188 C.Y. Tan, R.C. Lai, W. Wong, *et al.* Mesenchymal stem cell-derived exosomes promote hepatic regeneration in drug-induced liver injury models. *Stem Cell Res Ther* 2014;**5**:76.

189 Y.M. Yu, K.M. Gibbs, J. Davila, *et al.* MicroRNA miR-133b is essential for functional recovery after spinal cord injury in adult zebrafish. *Eur J Neurosci* 2011;**33**:1587–1597.

190 H. Xin, Y. Li, Z. Liu, *et al.* MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013;**31**:2737–2746.

191 H. Xin, Y. Li, B. Buller, *et al.* Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 2012;**30**:1556–1564.

192 H. Xin, Y. Li, Y. Cui, *et al.* Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013;**33**:1711–1715.

193 R.P. Stroemer, T.A. Kent, C.E. Hulsebosch. Neocortical neural sprouting, synaptogenesis, and behavioral recovery after neocortical infarction in rats. *Stroke* 1995;**26**:2135–2144.

194 Y. Li, J. Chen, M. Chopp. Adult bone marrow transplantation after stroke in adult rats. *Cell Transplant* 2001;**10**:31–40.

195 S.S. Lin, B. Zhu, Z.K. Guo, *et al.* Bone marrow mesenchymal stem cell-derived microvesicles protect rat pheochromocytoma PC12 cells from glutamate-induced injury via a PI3K/Akt dependent pathway. *Neurochem Res* 2014;**39**:922–931.

196 C. Lee, S.A. Mitsialis, M. Aslam, *et al.* Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 2012;**126**:2601–2611.

197 J.Y. Chen, R. An, Z.J. Liu, *et al.* Therapeutic effects of mesenchymal stem cell-derived microvesicles on pulmonary arterial hypertension in rats. *Acta Pharmacol Sin* 2014;**35**:1121–1128.

198 A.V. Vlassov, S. Magdaleno, R. Setterquist, R. Conrad. Exosomes: current knowledge of their composition, biological functions, diagnostic and therapeutic potentials. *Biochim Biophys Acta* 2012;**1820**:940–948.

199 R.W. Yeo, R.C. Lai, B. Zhang, *et al.* Mesenchymal stem cell: an efficient mass producer of exosomes for drug delivery. *Adv Drug Deliv Rev* 2013;**65**:336–341.

200 L. Kordelas, V. Rebmann, A.K. Ludwig, *et al.* MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014;**28**:970–973.

201 S. Dai, D. Wei, Z. Wu, *et al.* Phase I clinical trial of autologous ascites-derived exosomes combined with GM-CSF for colorectal cancer. *Mol Ther* 2008;**16**:782–790.

202 B. Escudier, T. Dorval, N. Chaput, *et al.* Vaccination of metastatic melanoma patients with autologous dendritic cell (DC) derived-exosomes: results of the first phase I clinical trial. *J Transl Med* 2005;**3**:10.

203 M.A. Morse, J. Garst, T. Osada, *et al.* A phase I study of dexosome immunotherapy in patients with advanced non-small cell lung cancer. *J Transl Med* 2005;**3**:9.

CHAPTER 39

# Evaluating mesenchymal stem/stromal cells for treatment of asthma and allergic rhinitis

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## 39.1 Summary

Asthma is a disease marked by reversible airway obstruction, persistent airway inflammation, and airway hyperresponsiveness. The remodeling of asthmatic airways is a complex process that represents a safe method of preventing chronic inflammation. Mesenchymal stem/stromal cells (MSCs) have been overwhelmingly shown to suppress inflammation. MSCs can be licensed to become immune suppressor cells within an inflamed microenvironment. Such an environment can be established by allergen-mediated airway contraction, edema, and mucus secretion. However, the molecular and cellular pathways by which MSCs suppress inflammation is complex and may depend on the cause of the inflammation. Specifically, experimental evidence has shown different responses by MSCs in the milieu created by activated T cells from allergic asthma and allergic rhinitis. This chapter discusses the complex inflammation caused by allergic diseases. We argue for more robust studies to effectively use MSCs. Another major unanswered question is whether a specific source of MSCs would be more effective in the treatment of allergic diseases.

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## 39.2 Introduction

Asthma is a disease marked by reversible airway obstruction, persistent airway inflammation, and airway hyperresponsiveness. The World Health Organization reported that approximately 235 million people suffer from asthma, spanning high-, middle-, and low-income countries. Asthma is a major public health concern and is still underdiagnosed and undertreated. It is a heterogeneous disease with many physiologic and clinical phenotypes. Allergic asthma is triggered by exposure to allergens, the effects of which are mediated primarily via pathways involving T helper (Th)2 cells, mast cells, and eosinophils. Nonallergic asthma, associated with nonallergen triggers such as fumes, typically do not involve the Th2 pathway. This will be discussed further later. The development of allergic diseases involves interplay between genetic predispositions and environmental exposures to triggers. The expanding insights into the mechanisms underlying asthma pathology have resulted in a broadening of available therapeutic modalities.

## 39.3 Early and late asthma response

Key mediators have been described in the pathogenesis of early inflammatory response as well as for late and chronic stages of asthma. Mediators such as histamine, leukotriene $D_4$ and $E_4$, and

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prostaglandin $D_2$ can mediate the early phase of asthma, which manifests as bronchospasm. Increased vascular permeability allows cells to leave the bloodstream and enter pulmonary tissue, thereby contributing further to inflammation.

Mast cells are one of the cells of the innate immune system implicated in asthma pathogenesis. Antigens cause crosslinking of the high-affinity FcεRI receptors on mast cells, resulting in degranulation and release of preformed inflammatory cytokines such as histamine and tryptase, as well as newly synthesized inflammatory mediators that include leukotriene $C_4$, prostaglandin $D_2$, platelet activating factor, and cytokines. Basophils, through their immunoglobulin (Ig)E receptors, can enable cross-linking by allergens to release histamine and leukotriene mediators. Basophils also secrete interleukin (IL)-4, which can cause Th2 differentiation. Innate lymphoid cells, sharing the same progenitor as T cells, are capable of skewing the immune response. Specifically, innate lymphoid cells 2 express IL-13 that can further promote Th2-driven inflammation. The late/chronic response is mediated by Th2 polarization, along with eosinophil- and neutrophil-mediated cytokine inflammation [1].

## 39.4 Airway remodeling

Asthmatic airway remodeling is a complex process that is the result of chronic inflammation. Allergen exposure and subsequent release of mediators cause airway contraction, edema, and mucus secretion. Chronic damage to the epithelial layer develops over time and exaggerates extracellular matrix deposition, causing subepithelial fibrosis and thickening of the basement membrane [2]. An imbalance in cytokine signaling results in altered extracellular matrix composition and increased smooth muscle. This results in abnormal airway remodeling that causes airflow obstruction and increased airway hyperresponsiveness. Additionally, smooth muscle hypertrophy serves as a nidus of proinflammatory cytokine production as well as mechanical bronchoconstriction. The process of basement membrane thickening occurs via increased collagen deposition. Collagen, produced by fibroblasts, is the primary component of the basement membrane. Metalloproteinase-9 can digest type IV collagen, thereby preventing excess collagen deposition. Tissue inhibitors of matrix metalloproteinase 1 (TIMP-1) interfere with metalloproteinase-9-mediated collagen degradation, resulting, as indicated earlier, in an exaggerated collagen deposition and thickening of the basement membrane. Transforming growth factor-β (TGF-β), synthesized by macrophages, lymphocytes, fibroblasts, airway epithelial cells, eosinophils, and mast cells, induces TIMP-1 expression. Furthermore, IL-13, a cytokine that is prominent in Th2-driven inflammation, promotes TIMP-1 production [3]. Cytokine-driven angiogenesis causes airway edema, which is a known contributor of asthma fatalities. Vascular endothelial growth factor is responsible for increased angiogenesis in asthmatic airways and is thought to be able to modulate smooth muscle cell proliferation [4].

## 39.5 Innate immunity of the airway

The airway is a rich repository of immune cells. Epithelial cells, monocytes, mast cells, neutrophils, and eosinophils are the innate immunity's sentinels in host defense (see also Chapter 33). Lymphocytes, members of the adaptive immune response, can mount antigen-specific defense. A fine balance between both arms of the immune system is necessary for mounting and maintaining a controlled immune response. A dysregulation of these systems results in asthmatic disease.

Epithelial cells lining the airway serve a physical barrier function to prevent invasion of pathogens and allergen sensitization. A compromised epithelial layer can allow allergen presentation to antigen-presenting cells (APCs) that can then sensitize the host's immune response. Additionally, epithelial cells secrete important inflammatory mediators. Thymic stromal lymphopoietin (TSLP) is an epithelial-derived cytokine that upregulates the APC function of dendritic cells in the Th2 pathway, thereby driving Th2-mediated inflammation [5].

Macrophages, another subset of innate immune cells, serve as an important cellular component in airway function. Macrophage-derived cytokines can polarize the immune response to Th1 or Th2 pathways. The M1 class of macrophages, when stimulated by interferon (IFN)-γ, causes a Th1 response. In contrast, M2 macrophages, when activated by IL-4 and IL-13, result in a Th2 response, which is a central pathway for allergic inflammation [6]. Of note, macrophages are sources of IL-10, which can suppress inflammation and promote the expansion of regulatory T cells (Tregs) [7].

In the lungs, dendritic cells can be located at the basolateral side of epithelial cells and sample luminal antigens. After antigen uptake, the dendritic cells migrate to the draining lymph nodes where they present the antigens to T cells. Dendritic cells can support Th2 polarization by decreasing Th1 and Th17, resulting in the production of IL-12, TGF-β, IL-23, and IL-6 [8].

Granulocytes are also an important component of airway physiology. There are few resident mast cells within the airways. Mast cells release both preformed mediators, such as histamine and tryptase, as well as newly synthesized mediators, such as bradykinin, in response to stimuli. Mast cells also secrete IL-4, IL-5, IL-9, IL-13, TSLP, and tumor necrosis factor (TNF)-α. Mast cells are a prominent source of vascular endothelial growth factor and fibroblast growth factor-2, promoting airway remodeling and edema [9]. Eosinophils are recruited to the airway during inflammation. *In vitro* studies have shown that eosinophils can promote IL-17 production through the release of IL-1β. As will be discussed further, IL-17 is an important mediator of inflammation in asthmatic airways [10]. Although eosinophil-mediated killing of invasive pathogens can serve a useful function, their recruitment during an allergic response can result in the release of cationic proteins. The release of proteins by eosinophils has been attributed to physiological and inflammatory damage seen in asthmatic airways.

## 39.6 Adaptive immunity of the respiratory tract

Epithelial integrity is one the foremost methods for maintaining intact tolerance. If compromised, this can predispose to the development of allergic disease. Frequent viral and bacterial infections can lead to damage of the epithelial membrane. Allergens can also compromise the integrity of the airway epithelial membrane, since certain allergens, such as dust mites' Der p 1, have proteolytic activity that can enzymatically cleave the epithelial layer. Der p 1, a cysteine protease found in dust mites, can degrade cellular adhesion molecules to cause mucosal permeability [11].

Allergen-mediated stimulation of the innate immune system can trigger an inflammatory response. Activation of dendritic cells caused by presentation of allergens can activate both the innate and adaptive immune systems. The allergic antigens are presented within the groove of the major histocompatibility complex (MHC) of dendritic cells. Co-stimulation through CD80 or CD86 on the dendritic cells is critical for naive T cells to be activated. These co-receptors interact with CD28 on T cells. The ensuing response (Th1 versus Th2) is determined by the cytokine milieu. As an example, IL-12 will favor Th1 differentiation and IL-4 will favor Th2 differentiation.

Anergy can occur when T cells are presented with the antigen–MHC complex in the absence of co-stimulation. This is a major mechanism through which tolerance occurs. In our *in vitro* studies, small amounts of MSCs with a low dose of allergen when added intermittently to the cultures resulted in tolerance when the T cells were washed and then re-exposed to the optimal dose of the allergen [12]. Th2 polarization is the predominant mechanism underlying allergic asthma inflammation. IL-25 and IL-33 produced by airway epithelial cells activate lymphoid cells that can then produce IL-4, IL-5, and IL-13, which are the primary cytokines driving allergic inflammation and promoting Th2 production. IL-4 and IL-13 also induce IgE class switch in B cells. IL-13 promotes airway hyperresponsiveness. Although Th2 is the predominant lymphocyte responsible for asthma pathology, other T cell types have been implicated in the disease. Th1 cells have been identified in chronic exacerbations. Th9 cells, the differentiation of which is mediated by IL-4 and TFG-β, increase mucus hypersecretion in asthmatic patients and further promotes Th2 activation [13]. Th17 cells secrete the proinflammatory cytokines IL-17 and IL-22 as well as IL-8, a neutrophil chemotactic factor. IL-17 promotes goblet cell hyperplasia and smooth muscle contraction. IL-22 can contribute to smooth muscle proliferation and subsequent airway remodeling [14]. Although the role of neutrophils in asthma is not yet well defined, a predominance of neutrophils in asthma results in more severe asthma, and the presence of atopy does not exclude this non-eosinophilic asthma variant [15]. (Atopy (atopic syndrome) is a syndrome characterized by a tendency to be "hyperallergic." A person with atopy typically presents with one or more of the following: eczema (atopic dermatitis), allergic rhinitis (hay fever), or allergic asthma.)

## 39.7 Toll-like receptors

Inflammation is characterized by redness, pain, swelling, heat and loss of tissue function. It is a protective response to various factors including microbial infection and tissue injury [16,17]. The symptoms related to inflammation increase vascular endothelium permeability, resulting in access for immune cells. The major contributor to acute inflammation induced by microbial infection or tissue damage is caused by the innate immune response, commonly referred to as the first line of defense [16–20]. Germline-encoded pattern recognition receptors (PRRs) sense the presence of microorganisms by identifying microbial structures called pathogen-associated molecular patterns (PAMPs). One class of the PRR family is the transmembrane protein toll-like receptors (TLRs).

TLR-mediated defense is critical for the host's response to invading pathogens [16–19]. Eleven TLRs have been identified in humans. Their differential expression and regulation has led to the identification of various ligands [16]. Besides activated immune cells such as dendritic cells and macrophages, PRRs are also expressed on nonprofessional APCs (such as epithelial cells), and other cells, such as human MSCs, have been shown to express the intracellular receptor TLR3 and cell surface receptor TLR4, which recognize PAMPs' nucleic acid and lipopolysaccharide respectively [17]. The stimulation of human MSCs with lipopolysaccharide or poly(I:C) leads to the production of inflammatory mediators [21]. Expression of TLRs is further increased by stimulation of IFN-α and IFN-γ, resulting in the conversion of the MSCs to immune suppressor cells [21].

This section briefly discusses the role of MSCs as a therapeutic strategy for allergic responses. MSCs can modulate an immune response by direct regulation of the cellular response at the site of infection or injury [22]. In order to exert immunomodulatory properties, MSCs rely on intercellular communication, partly through the secretion of soluble factors such as autonomous cytokines and/or chemokines from neighboring activated immune cells [23]. The effects of MSCs on the innate and adaptive immune response can be mediated by the TLRs. Thus, these effects need to be considered when considering cellular therapies.

## 39.8 Allergic rhinitis and immunology

Allergic rhinitis is an inflammatory disease of the nasal mucosa characterized by nasal pruritus, sneezing, rhinorrhea, and nasal congestion [24]. It is associated with other inflammatory disorders, including asthma, rhinosinusitis, and allergic conjunctivitis. Although boys are more likely to have allergic rhinitis during childhood, this tendency reverses in puberty, and by adulthood both men and women are affected equally [25]. Atopy

is the abnormal development of specific IgE in response to harmless and ubiquitous environmental allergens. Allergic rhinitis is often associated with other atopic diseases, including allergic rhinoconjunctivitis, asthma, atopic dermatitis, and food allergies. These disorders have been linked to genetic loci on chromosomes 2, 5, 6, 7, 11, 13, 16, and 20 [26]. Allergic rhinitis has been traditionally classified as seasonal or perennial, based on sensitization to seasonal or year-round allergens. On exposure to an allergen, symptoms arise within minutes and can last 1–2 h before resolution as an immediate hypersensitivity reaction via mast cells and basophils. Late-phase symptoms include nasal obstruction, hyposmia, postnasal mucous discharge, and nasal hyper-reactivity [27].

Allergic conjunctivitis differentiates allergic rhinitis from other forms of rhinitis. It is characterized by intense itching, hyperemia, watering of the eyes, and periorbital edema. Pollen-induced allergic rhinitis can have an associated oral allergy syndrome, including oral and pharyngeal itchiness, tingling, erythema, and angioedema of the tongue, lip, and soft palate after oral contact with fruits and vegetables [28]. To determine the cause, a detailed history is necessary to identify potential triggers and reveal other allergic diseases. To confirm the diagnosis, either skin-prick testing or serum levels of specific IgE are used to record specific IgE reactivity to airborne allergens. Although IgE is the mainstay in the pathogenesis of allergic rhinitis, new developments have started to elucidate the underlying complex mechanism. This section will focus on the immunologic basis behind allergic rhinitis.

Individuals with allergic rhinitis are sensitized to an antigen via activation of dendritic cells and T lymphocytes [29]. Dendritic cells at the mucosal surfaces capture allergens and act as APCs to the T lymphocytes in the draining lymph nodes. $CD4^+$ T cells secrete cytokines, including ILs, to initiate and coordinate the allergic immune response. Regulatory T lymphocytes that express the surface markers CD4 and CD25, and Tr1 cells that produce IL-10, normally suppress the Th2 response to allergens, but this inhibition is decreased in allergic diseases [29]. IL-4 and IL-13, along with CD40 ligation, induce IgE class switch in B lymphocytes, driving sensitization to allergens [30]. IgE molecules are released into the bloodstream and bind to mast cells and basophils. When an allergen binds to surface IgE on mast cells in the nasal mucosa, there is a rapid release of preformed mediators such as histamine, which cause symptoms associated with early allergic response. Histamine, along with TNF-α, leukotriene $C_4$, and prostaglandin $D_2$, stimulate the expression of adhesion molecules on endothelial cells and contribute to the influx of inflammatory cells comprised of eosinophils, $CD4^+$ T lymphocytes, and basophils. The influx of these inflammatory cells creates the late allergic response [29].

IgE is a well-known mediator of allergic reactions, including allergic rhinitis and asthma. It differs from other antibodies as it is primarily located in the tissues, tightly bound to mast cells and basophils via a high-affinity receptor. IgE does not bind complement and functions mainly through its receptors. It can be produced in the regional lymph nodes or bone marrow, as well as locally in the mucosa, by plasma cells. At least half of patients with allergic rhinitis have positive skin tests, and a higher degree of peripheral eosinophilia is associated with an increased risk of more severe sinus disease [31,32]. However, a positive allergy test does not necessary prove a causal relationship with allergic rhinitis [33]. Based on previous studies, there is insufficient evidence to associate classical IgE-mediated allergy as the major mechanism behind allergic rhinitis [34].

In contrast, there is little disagreement with local inflammation and production of IgE in allergic rhinitis. The concentration of IgE in nasal secretions was higher than in serum [35]. After intranasal exposure to an allergen, specific IgE was elevated in nasal washings, suggesting local intranasal IgE production [36,37]. In addition, secondary lymphoid follicles with T cells, B cells, and plasma cells associated with IgE were found in nasal tissue [36]. ILs and CD40, elements needed for IgE production, were discovered in the sinonasal mucosa [35]. Heavy-chain class-switching and IgE induction in response to antigen was also demonstrated in the sinonasal tissue, further supporting local production of IgE [38]. IgE also plays a central role in immediate hypersensitivity. Cross-linking of bound IgE with an allergen on mast cells and basophils triggers degranulation and the release of inflammatory mediators. IgE receptors on dendritic cells facilitate the presentation of allergens to T cells, and low-affinity IgE receptors on B cells augment cellular and humoral immune responses [39].

B cells are integral to IgE production and allergen sensitization via IgE class switching. A subset, B regulatory cells 1 (Br1), produce high amounts of IL-10, inhibiting proliferation and differentiation of Th1 and Th2 cells and altering TNF expression in effector $CD4^+$ T cells. Activated Bregs can enhance Treg properties by increasing Foxp3 expression and increase the suppressive co-receptor, cytotoxic T lymphocyte antigen 4 [40]. Semaphorin3A (sema3A) is a soluble molecule involved in immune system modulation and maintenance of self-tolerance. It is present on Tregs as a suppressor marker. In addition, sema3A was found on Bregs, along with its receptor NP-1 and CD72, a regulatory molecule on B cells that inhibits B cell receptor pathway activation signals. In allergic rhinitis there is hyperinnervation of sensory neurons in the nasal turbinate, possibly associated with itching and sneezing. Sema3A inhibits sprouting of nerve fibers and restricts innervation of sensory neurons. There is a decreased expression of sema3A in the nasal epithelium, accompanied by an increase in nerve fiber density in a murine allergic rhinitis model. Treating the mice with recombinant sema3A, a possible therapeutic option, decreased the density of nerve fibers compared with untreated mice with allergic rhinitis [41].

Accumulation of basophils is present in allergic inflammation. Basophils are myeloid hematopoietic cells. Basophil maturation is not well understood, but transcription factors, microbial-derived signals, and IgE regulate their development. Th2 cytokine-mediated inflammatory responses, via IL-3 and thymic stromal lymphopoietin, increase basophil development, and cause peripheral basophilia. Basophils can be activated through

multiple signals, including cytokines, antibodies, proteases and directly by antigens. Local infiltration of immune cells into the nasal mucosa of patients with allergic rhinitis includes basophils. Basophils are a major source of histamine in the late-phase responses to allergen challenge. They also provide leukotriene $C_4$, important in the activation of mast cells [42].

Cytokines are major mediators in the pathogenesis of allergic rhinitis. Cytokines can be produced by immune, structural, and other cells within an organ, where they coordinate inflammatory responses. The effects of cytokines can be triggered by any insult, but are exacerbated by infection, trauma, allergic challenges, and autoimmune diseases, among others. In allergic rhinitis, a Th2-mediated response with associated cytokines such as IL-4, IL-5, and IL-13 are elevated in the nasal mucosa [43]. The levels of these cytokines generally correlate with symptom severity [43]. IL-10, a regulatory cytokine, is also increased in nasal fluid after an allergen challenge. The Th2-linked chemokines, eotaxin, RANTES (regulation on activation, normal T cell expressed and secreted and also known as CCL5), monocyte chemoattractant protein 1 (also known as CCL2), and macrophage inflammatory protein 1α, are reportedly increased in allergic rhinitis. The Th1-mediated inflammatory cytokine IFN-γ was not significantly increased in nasal fluid after an allergen challenge. The role of IL-17 in allergic rhinitis is unclear, and its elevation, may depend on the specific allergen, such as dust mite. Epithelial-derived cytokines, TSLP, IL-33, and IL-25 are released by allergen exposure and affect Th2 cells directly or via innate lymphoid cells that produce cytokines to activate Th2 cells. The role of epithelial-derived cytokines in allergic rhinitis is unclear, and studies have been inconclusive to date [44]. IL-33 is released upon structural cell damage and activates multiple immune cells, leading to the production of Th2-like cytokines. It has been postulated that IL-33 exacerbates allergic rhinitis via the activation of mast cells to produce proinflammatory cytokines and chemokines [45].

The innate immune system has an important role in allergic rhinitis. The sinonasal epithelium is an interface for pathogens via their PAMPs, and the innate immune system via PRRs. TLRs have a dual role in allergic diseases. Some cause sensitization and break tolerance, while others promote tolerance to allergens. TLRs impact adaptive immunity by deviating Th2 to Th1 and maturation of Tregs [35]. TLR9, a bacterial DNA receptor in the nasal mucosa, is decreased in allergic rhinitis [46]. Given its role in the pathogenesis of allergic rhinitis, TLR9 has been the aim of research as a possible therapeutic target to block TLR9-mediated immune activation [47]. TLR4 is upregulated in allergic rhinitis, and the goal of targeting TLR4 using adjuvants of allergy vaccines is to induce tolerance and the inhibition of its inflammatory function [48].

## 39.9 Immune modulation by mesenchymal stem/stromal cells

MSCs first emerged as immune-related cells when it was observed that MSCs inhibited T cell proliferation *in vitro* [49]. This observation was validated *in vivo*, when MSCs were co-administered during the transplantation of allogeneic bone marrow hematopoietic stem cells and progenitors. MSCs were given to reduce the risk of graft rejection, a recipient T cell-mediated host-versus-graft response [50]. They have more commonly been used to treat or prevent graft-versus-host disease, another complication of allogeneic hematopoietic stem cell transplantation, in this case mediated by donor T cells reacting against recipient (host) tissues (see Chapter 57). These studies led to further research on the broader immunomodulatory properties of MSCs. Over the past decade, MSCs have been found capable of exerting either immune-suppressing or immune-enhancing functions (reviewed in [51] and [52]), as well as releasing various cytokines capable of acting in an autocrine or paracrine fashion [53].

MSCs have been shown to suppress T cell proliferation [49,54]. Although the exact mechanism is unknown, MSCs induce cell cycle arrest in the $G_0/G_1$ phase in T cells, possibly through the production of indoleamine-pyrrole 2,3-dioxygenase (IDO), prostaglandin $E_2$ ($PGE_2$), or by the induction of the inhibitory molecule programmed cell death protein 1 (PD-1, also known as CD279) [55,56]. MSCs do not always act as immune suppressor cells. They can act as APCs [57]. In other studies, MSCs have been shown to lack the co-stimulatory molecules CD80 and CD86 that are needed to stimulate T cells together with specific antigen. Because of this, MSCs could lead to T cell anergy [58]. MSCs can secrete cytokines to influence T cell differentiation. These include hepatocyte growth factor, IL-6, IL-10, and TGF-β1 [59]. Specifically, MSCs can suppress the differentiation into Th1 and Th17 cells and enhance Th2 and Treg differentiation [60].

MSCs can arrest B cells in the $G_0/G_1$ phase of the cell cycle, and further reduce B cell chemotaxis [61]. The mechanism of B cell arrest is unclear, but may be based on the PD-1 pathway [55], as reported for T cell arrest, or could occur from MSC-mediated production of soluble factors [61]. Conversely, some studies have shown that MSCs can stimulate B cell proliferation and differentiation [62].

MSC-derived IDO and $PGE_2$ have major roles in the suppression of MSCs on natural killer (NK) cell proliferation [63,64]. In addition, MSCs can further inhibit NK cell activity by down-regulating the NK-activating receptors NKG2D, NKp30, and NKp44 [64]. Down-modulation of these activating receptors involves reduced production of proinflammatory cytokines, resulting in suppression of NK cell function [64]. MSCs inhibit dendritic cell differentiation and maturation *in vitro*. Studies have shown that this occurs through the production of $PGE_2$ by MSCs [65].

Macrophages normally differentiate into proinflammatory M1 or anti-inflammatory M2 subtypes. In the presence of MSCs, macrophages selectively differentiate into the anti-inflammatory M2 macrophage subtype [66,67]. Again, the IDO and $PGE_2$ pathways have been implicated in this anti-inflammatory modulation, as has the MSC-secreted cytokine IL-10, among other cytokines [68].

MSCs have been shown to suppress neutrophil infiltration [69,70]. IL-10 and TGF-β secreted by MSCs reduce E-

selectins on endothelial cells to minimize the extravasation of neutrophils [70]. Meanwhile, MSCs secrete IL-6, IL-8, and macrophage inhibitory protein [71], which have been implicated in the activation of neutrophils, while IL-6 also inhibits neutrophil respiratory bursts [72], thus suppressing neutrophil function and subsequent tissue damage.

## 39.10 The future of mesenchymal stem/stromal cells as therapy for allergic diseases

MSCs appear to be primarily anti-inflammatory effector cells, making them an ideal candidate for cell therapy. This immune suppression relies on multiple pathways and secreted factors. Before transitioning MSCs to the clinic, however, we must more fully understand the microenvironment in which these cells will find themselves, and how that environment will act on the MSCs. Experimental evidence has shown different responses by MSCs in the milieu created by activated T cells in allergic asthma compared with allergic rhinitis [12,73]. The brief discussion in this chapter shows that allergic diseases are complex. Despite the inflammatory milieu formed by allergic disease, experimental evidence indicates that the triggers cause different outcomes in MSCs. Our studies used bone-marrow-derived MSCs. The results indicate that the molecular and cellular methods by which allergic rhinitis and allergic asthma triggered MSCs to respond might be different. These findings indicate that additional research studies are required before introducing MSCs to the clinic for these two allergic disorders.

Because of their immune modulatory properties, MSCs remain a good candidate for the cellular treatment of allergic diseases. Additionally, since MHC matching between MSC donors and MSC recipients does not appear to be required, banks of allogeneic MSCs from a number of unrelated donors can be cryopreserved, allowing direct access to MSCs as needed. Thus far, there have been no overt pathological problems reported from the use of allogeneic MSCs for inflammatory diseases. However, the pathogenesis of inflammatory airway diseases, such as allergic asthma and allergic rhinitis, are complex, although there is some overlap due to the involvement of the innate and adaptive immune responses in each. (Figure 39.1).

It will be important to understand what happens to MSCs when they act as immune-suppressive cells, how they will react to the local environment in which they are placed, and what happens when the inflammation becomes chronic.

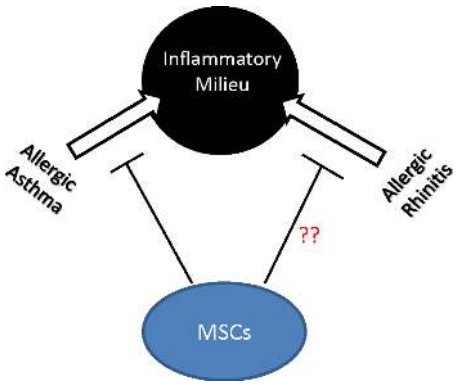



**Figure 39.1** Shown are seemingly similar inflammatory milieu after an allergic response by subjects with asthma and rhinitis. The experimental results thus far show that MSCs can induce tolerance and reverse the inflammation in allergic asthma [12]. However, a similar response has not been noted for allergic rhinitis [73]. The "??" depicts an unknown method by which MSCs can be used for allergic rhinitis.

## References

1 Hall S, Agrawal DK. Key mediators in the immunopathogenesis of allergic asthma. *Int Immunopharmacol* 2014;**23**:316–329.
2 Reeves SR, Kolstad T, Lien TY, *et al.* Asthmatic airway epithelial cells differentially regulate fibroblast expression of extracellular matrix components. *J Allergy Clin Immunol* 2014;**134**:663–670.
3 Yamauchi K, Inoue H. Airway remodeling in asthma and irreversible airflow limitation – ECM deposition in airway and possible therapy for remodeling. *Allergol Int* 2007;**56**:321–329.
4 Berraies A, Hamzaoui K, Hamzaoui A. Link between vitamin D and airway remodeling. *J Asthma Allergy* 2014;**7**:23–30.
5 Watson B, Gauvreau GM. Thymic stromal lymphopoietin: a central regulator of allergic asthma. *Expert Opin Ther Targets* 2014;**18**: 771–785.
6 Moreira AP, Hogaboam CM. Macrophages in allergic asthma: fine-tuning their pro- and anti-inflammatory actions for disease resolution. *J Interferon Cytokine Res* 2011;**31**:485–491.
7 Belardelli F. Role of interferons and other cytokines in the regulation of the immune response. *APMIS* 1995;**103**:161–179.
8 Van Helden MJ, Lambrecht BN. Dendritic cells in asthma. *Curr Opin Immunol* 2013;**25**:745–754.
9 Kearley J, Erjefalt JS, Andersson C, *et al.* IL-9 governs allergen-induced mast cell numbers in the lung and chronic remodeling of the airways. *Am J Respir Crit Care Med* 2011;**183**:865–875.
10 Esnault S, Kelly EAB, Nettenstrom LM, *et al.* Human eosinophils release IL-1β and increase expression of IL-17A in activated $CD4^+$ T lymphocytes. *Clin Exp Allergy* 2012;**42**:1756–1764.
11 Kauffman HF. Interaction of environmental allergens with airway epithelium as a key component of asthma. *Curr Allergy Asthma Rep* 2003;**3**:101–108.
12 Kapoor S, Patel SA, Kartan S, *et al.* Tolerance-like mediated suppression by mesenchymal stem cells in patients with dust mite allergy-induced asthma. *J Allergy Clin Immunol* 2012;**129**: 1094–1101.
13 Xing J, Wu Y, Ni B. Th9: a new player in asthma pathogenesis? *J Asthma* 2011;**48**:115–125.
14 Farahani R, Sherkat R, Hakemi MG, *et al.* Cytokines (interleukin-9, IL-17, IL-22, IL-25 and IL-33) and asthma. *Adv Biomed Res* 2014;**3**:127.
15 Moore WC, Hastie AT, Li X, *et al.* Sputum neutrophil counts are associated with more severe asthma phenotypes using cluster analysis. *J Allergy Clin Immunol* 2014;**133**:1557–1563.

16 Takeuchi O, Akira S. Pattern recognition receptors and inflammation. *Cell* 2010;**140**:805–820.
17 Kawai T, Akira S. Toll-like receptors and their crosstalk with other innate receptors in infection immunity. *Immunity* 2011;**34**:637–650.
18 Vasselon T, Detmers PA. Toll receptors: a central element in innate immune responses. *Infect Immun* 2002;**70**:1033–1041.
19 Brennan CA, Anderson KV. *Drosophila*: the genetics of innate immune recognition and response. *Annu Rev Immunol* 2004;**22**: 457–483.
20 Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat Immunol* 2001;**2**:675–680.
21 Romieu-Mourez R, Francois M, Boivin MN, *et al*. Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. *J Immunol* 2009;**182**:7963–7973.
22 Delarosa O, Dalemans W, Lombardo E. Toll-like receptors as modulators of mesenchymal stem cells. *Front Immunol* 2012;**3**:182.
23 Patel S, Sherman L, Munoz J, Rameshwar P. Immunological properties of mesenchymal stem cells and clinical implications. *Arch Immunol Ther Exp* 2008;**56**:1–8.
24 Shusterman D. Occupational irritant and allergic rhinitis. *Curr Allergy Asthma Rep* 2014;**14**:1–8.
25 Bousquet J, van Cauwenberge P, Khaltaev N. Allergic rhinitis and its impact on asthma. *J Allergy Clin Immunol* 2001;**108**:S147–S334.
26 Brunekreef B, Smit J, De Jongste J, *et al*. The Prevention and Incidence of Asthma and Mite Allergy (PIAMA) birth cohort study: design and first results. *Ped Allergy Immunol* 2002;**13**:55–60.
27 Wahn U, Bergmann RL, Nickel R. Early life markers of atopy and asthma. *Clin Exp Allergy* 1998;**28**:20–21.
28 Callebaut I, Spielberg L, Hox V, *et al*. Conjunctival effects of a selective nasal pollen provocation. *Allergy* 2010;**65**:1173–1181.
29 Greiner AN, Hellings PW, Rotiroti G, Scadding GK. Allergic rhinitis. *Lancet* 2011;**378**:2112–2122.
30 Dullaers M, De Bruyne R, Ramadani F, *et al*. The who, where, and when of IgE in allergic airway disease. *J Allergy Clin Immunol* 2012;**129**:635–645.
31 Emanuel IA, Shah SB. Chronic rhinosinusitis: allergy and sinus computed tomography relationships. *Otolaryngol Head Neck Surg* 2000;**123**:687–691.
32 Newman LJ, Platts-Mills TE, Phillips C, *et al*. Chronic sinusitis: relationship of computed tomographic findings to allergy, asthma, and eosinophilia. *J Am Med Assoc* 1994;**271**:363–367.
33 Rosenfeld RM, Andes D, Neil B, *et al*. Clinical practice guideline: adult sinusitis. *Otolaryngol Head Neck Surg* 2007;**137**:S1–S31.
34 Pant H, Ferguson BJ, Macardle PJ. The role of allergy in rhinosinusitis. *Curr Opin Otolaryngol Head Neck Surg* 2009;**17**:232–238.
35 Hoddeson EK, Pratt E, Harvey RJ, Wise SK. Local and systemic IgE in the evaluation and treatment of allergy. *Otolaryngol Clin North Am* 2010;**43**:503–520.
36 Smurthwaite L, Durham SR. Local IgE synthesis in allergic rhinitis and asthma. *Curr Allergy Asthma Rep* 2002;**2**:231–238.
37 Merrett TG, Houri M, Mayer AL, Merrett J. Measurement of specific IgE antibodies in nasal secretion – evidence for local production. *Clin Allergy* 1976;**6**:69–73.
38 Takhar P, Smurthwaite L, Coker HA, *et al*. Allergen drives class switching to IgE in the nasal mucosa in allergic rhinitis. *J Immunol* 2005;**174**:5024–5032.
39 Gelfand EW. Inflammatory mediators in allergic rhinitis. *J Allergy Clin Immunol* 2004;**114**:S135–S138.
40 Kessel A, Haj T, Peri R, *et al*. Human $CD19^{+}CD25^{high}$ B regulatory cells suppress proliferation of $CD4^{+}$ T cells and enhance Foxp3 and CTLA-4 expression in T-regulatory cells. *Autoimmunity Rev* 2012;**11**:670–677.
41 Vadasz Z, Haj T, Toubi E. The role of B regulatory cells and semaphorin3A in atopic diseases. *Int Arch Allergy Immunol* 2014;**163**: 245–251.
42 Siracusa MC, Kim BS, Spergel JM, Artis D. Basophils and allergic inflammation. *J Allergy Clin Immunol* 2013;**132**:789–801.
43 Michaud B, Gouvis-Echraghi R, Candon S, *et al*. Quantification of circulating house dust mite-specific IL-4- and IL-13-secreting T cells correlates with rhinitis severity in asthmatic children and varies with the seasons. *Clin Exp Allergy* 2014;**44**:222–230.
44 Scadding G. Cytokine profiles in allergic rhinitis. *Curr Allergy Asthma Rep* 2014;**14**:1–8.
45 Saluja R, Ketelaar ME, Hawro T, *et al*. The role of the IL-33/IL-1RL1 axis in mast cell and basophil activation in allergic disorders. *Mol Immunol* 2015;**63**:80–85.
46 Melvin TA, Ramanathan M, Jr. Role of innate immunity in the pathogenesis of allergic rhinitis. *Curr Opin Otolaryngol Head Neck Surg* 2012;**20**:194–198.
47 Matesic D, Lenert A, Lenert P. Modulating Toll-like receptor 7 and 9 responses as therapy for allergy and autoimmunity. *Curr Allergy Asthma Rep* 2012;**12**:8–17.
48 Aryan Z, Holgate ST, Radzioch D, Rezaei N. A new era of targeting the ancient gatekeepers of the immune system: toll-like agonists in the treatment of allergic rhinitis and asthma. *Int Arch Allergy Immunol* 2014;**164**:46–63.
49 Di Nicola M, Carlo-Stella C, Magni M, *et al*. Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:3838–3843.
50 Le Blanc K, Samuelsson H, Gustafsson B, *et al*. Transplantation of mesenchymal stem cells to enhance engraftment of hematopoietic stem cells. *Leukemia* 2007;**21**:1733–1738.
51 Marigo I, Dazzi F. The immunomodulatory properties of mesenchymal stem cells. *Semin Immunopathol* 2011;**33**:593–602.
52 Sherman LS, Munoz J, Patel SA, *et al*. Moving from the laboratory bench to patients' bedside: considerations for effective therapy with stem cells. *Clin Transl Sci* 2011;**4**:380–386.
53 Castillo M, Liu K, Bonilla L, Rameshwar P. The immune properties of mesenchymal stem cells. *Int J Biomed Sci* 2007;**3**:76–80.
54 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2004;**105**:1815–1822.
55 Augello A, Tasso R, Negrini S, *et al*. Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;**35**:1482–1490.
56 Ghannam S, Bouffi C, Djouad F, *et al*. Immunosuppression by mesenchymal stem cells: mechanisms and clinical applications. *Stem Cell Res Ther* 2010;**1**:2.
57 Stagg J, Pommey S, Eliopoulos N, Galipeau J. Interferon-γ-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2005;**107**:2570–2577.
58 Glennie S, Soeiro I, Dyson PJ, *et al*. Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2004;**105**:2821–2827.

59 Baraniak PR, McDevitt TC. Stem cell paracrine actions and tissue regeneration. *Regen Med* 2010;**5**:121–143.
60 Li G, Yuan L, Ren X, *et al*. The effect of mesenchymal stem cells on dynamic changes of T cell subsets in experimental autoimmune uveoretinitis. *Clin Exp Immunol* 2013;**173**:28–37.
61 Corcione A, Benvenuto F, Ferretti E, *et al*. Human mesenchymal stem cells modulate B-cell functions. *Blood* 2005;**107**:367–372.
62 Traggiai E, Volpi S, Schena F, *et al*. Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008;**26**:562–569.
63 Pradier A, Passweg J, Villard J, Kindler V. Human bone marrow stromal cells and skin fibroblasts inhibit natural killer cell proliferation and cytotoxic activity. *Cell Transplant* 2011;**20**:681–691.
64 Spaggiari GM, Capobianco A, Abdelrazik H, *et al*. Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin $E_2$. *Blood* 2008;**111**:1327–1333.
65 Spaggiari GM, Abdelrazik H, Becchetti F, Moretta L. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin $E_2$. *Blood* 2009;**113**:6576–6583.
66 Abumaree MH, Al Jumah MA, Kalionis B, *et al*. Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages. *Stem Cell Rev Rep* 2013;**9**:620–641.
67 Cho D-I, Kim MR, Jeong H-y, *et al*. Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Exp Mol Med* 2014;**46**:e70.
68 Maggini J, Mirkin G, Bognanni I, *et al*. Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE* 2010;**5**:e9252.
69 Casey A, Dirks F, Liang OD, *et al*. Bone marrow-derived multipotent stromal cells attenuate inflammation in obliterative airway disease in mouse tracheal allografts. *Stem Cells Int* 2014;**2014**:468927.
70 Nemeth K, Leelahavanichkul A, Yuen PST, *et al*. Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.
71 Brandau S, Jakob M, Bruderek K, *et al*. Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes. *PLoS ONE* 2014;**9**:e106903.
72 Raffaghello L, Bianchi G, Bertolotto M, *et al*. Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 2008;**26**:151–162.
73 Desai MB, Gavrilova T, Liu J, *et al*. Pollen-induced antigen presentation by mesenchymal stem cells and T cells from allergic rhinitis. *Clin Transl Immunol* 2013;**2**:e7.

CHAPTER 40

# Stem cell therapies for Huntington's disease

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## 40.1 Introduction

Huntington's disease (HD) is a hereditary neurodegenerative disorder in which a mutation of a single gene leads to the progressive loss of neurons throughout the brain resulting in the loss of both cognitive and physical faculties. The onset of HD symptoms occurs at about 40 years of age, and these patients usually succumb to the disease approximately 20 years after the emergence of these symptoms. Currently, there is no treatment to delay the onset of the disease or to halt its progression. Early clinical and preclinical studies in which stem cells were transplanted into a degenerated environment have provided therapeutically relevant benefits, establishing a proof of principle in which stem cells or their progeny are a viable therapy. However, the optimal approach for cell therapy to treat HD has not been established. In this chapter we will first outline the multifaceted nature of HD. We will also describe the types of cells being studied, with a focus on their ability to protect against further degeneration, as well as which types of cells may be more suitable for replacing neurons. The disease was initially called Huntington's chorea. Choreiform movements are involuntary, quick, sudden, random, jerky, purposeless spasmodic movements that can occur in any part of the body, often in the shoulders, arms or hands, or in the face and tongue.

## 40.2 Huntington's disease

Written accounts of the characteristics of HD have been available for centuries. One of the first accounts of what researchers believe to be related to HD was in the 14th century, when the epidemic "dancing mania" was described. Around the turn of the 17th century English colonists in the Americas used the terms "that disorder" or "Saint Vitus's Dance" to describe the involuntary movements, which were believed to be a result of possession by the devil and possibly led to one execution during the Salem witch trials in the 1690s [1]. In 1872, Dr. George Huntington gave his paper "On Chorea" to the Mason Academy of Medicine in Middleport, Ohio, which was published in *The Medical and Surgical Reporter* 2 months later [2]. In this paper Huntington began by describing many of the already known features of chorea. However, apart from his predecessors, Huntington describes in great detail what he considered a strikingly different form of chorea based upon observations of a small community in

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Long Island, New York. This new form of chorea, according to Huntington, was hereditary in nature with patterns of inheritance, as well as being of adult onset and progressive in nature. Huntington also described the tendencies toward insanity, leading to suicide.

Having established HD as being hereditary in nature, it was not until the Venezuela Project when a more comprehensive understanding of the genetic patterns of HD was elucidated. Beginning with the discovery of an isolated population with an inordinately high frequency of HD on the shores of Lake Maracaibo in Venezuela [3], researchers were able to establish a pedigree of over 10 000 individuals, all stemming from a single ancestor. Various samples taken from these individuals helped to locate the DNA polymorphism situated on chromosome 4 [4].

It was not until 10 years after the discovery of the location of the mutation that a multi-institutional collaborative effort led to finding the exact gene, IT15 (now termed *huntingtin*; *HTT*), which is responsible for HD [5]. In addition, these investigators discovered that the mutation of this gene involved an increase in the numbers of CAG trinucleotide repeats on exon 1 [5]. The vast majority of cases of HD involve an expansion of CAG repeats of the *HTT* gene on one allele. Alleles can be classified as healthy, mutable, and penetrant [6]. Healthy alleles are ones in which fewer than 26 CAG repeats are observed and no symptoms will appear [5]. Individuals with a mutable allele (27–35 CAG repeats) will show no symptoms of HD but have the risk of passing a penetrant allele to their offspring [7]. Penetrant alleles can be broken down into reduced penetrance (36–39) or full penetrance (40 or more CAG repeats). Individuals with a reduced penetrant allele are at risk for developing HD but may never develop symptoms, while individuals with fully penetrant alleles are associated with fully developed symptoms [8].

Anticipation is also observed in HD, a phenomenon whereby successive generations show increased disease severity at a reduced age of onset, commonly by paternal transmission through CAG repeat instability during spermatogenesis [9]. The number of CAG repeats has also been suggested to play a role in the age of onset [8,10] as well as the variability of progression [11]. Individuals with a greater number of CAG repeats (>60 CAG) will show the less common Westphal variant (or juvenile onset) of HD, while fewer repeats are associated with adult onset. Similarly, higher CAG repeats are correlated with an increase in the deterioration of cognitive and motor function [12,13].

### 40.2.1 Prevalence and symptomology

Based on a meta-analysis of 13 different studies on the incidence of HD worldwide, Pringsheim *et al.* estimated the prevalence of HD in the western world at about 5.7 cases per 100 000 and a prevalence of 0.4 cases per 100 000 in Asia [14]. Apart from the Lake Maracaibo community, high frequencies of HD are also observed in certain regions of western Europe [15]. Approximately 5–10% of all cases of HD are of the Westphal variant, in which the onset of symptoms appears before the age of 20 [9,16].

The progression of HD is highly variable, with the severity of symptoms correlating with the age of onset [10]. This is usually between the ages of 35 and 40 in adult-onset HD. Whereas death of individuals with adult-onset HD is approximately 20 years after the initial emergence of symptoms, juveniles with the Westphal variant of HD (a hypokinetic rigid form of the disease) have a much shorter life expectancy [17].

Symptoms of HD can be characterized as motor, nonmotor, and hypothalamus-related dysfunction. The progression of HD symptoms can be divided into early, mid, and late stage. Prior to the self-reported symptom of involuntary movements, patients slowly merge from the healthy phase to a prediagnostic phase in which the first subtle symptoms appear. This early stage has been extensively studied through the Predict HD study, a multi-institutional collaborative study designed to elucidate the earliest signs of HD [18,19]. Reports from these studies show changes in olfactory sensation and finger-tapping motor scores one to two decades prior to diagnosis [18]. Between high-risk HD carriers and noncarriers, significant differences appear in verbal and logistical memory [18,20]. Included in these early-stage symptoms are clumsiness, irritability, delusions, and depression with ideations toward suicide [21].

Symptoms developing during mid-stage HD are increasingly motor in nature [6,22]. Involuntary movements and difficulty initiating movement emerge during this stage, as well as a continuation of cognitive impairments and emotional disturbances and increases in sleeping difficulties and sexual decline. The mid to end stages are characterized by increased weight loss, chorea, inability to maintain balance, general weakness, speech difficulty, and loss of bladder and bowel control [6,23]. As the progression of the disease continues, most patients suffer from rigidity and bradykinesia and lose the ability to care for themselves. They usually die due to complications from falls, dysphagia, or pneumonia, often as a result of difficulties in aspiration, such as inhaling a foreign substance (e.g., food) into the lungs [1,15].

### 40.2.2 Neuronal pathology

From a neuropathological perspective HD is unique, in that the mutation primarily leads to an early, massive degeneration of medium spiny neurons (MSNs) within the striatum, with most of the extrastriatal neuronal loss occurring in later stages of the disease [24,25]. The striatum is a structure within the interconnected nuclei of the basal ganglia, which includes the caudate nucleus, putamen, globus pallidus, substantia nigra, and thalamus. The striatum, also known as the neostriatum or striate nucleus, is a subcortical part of the forebrain. It receives input from the cerebral cortex and is the primary input to the basal ganglia system. In all primates, the striatum is divided by a white-matter tract called the internal capsule into two sectors called the caudate nucleus and the putamen (Figure 40.1).

Functionally, the striatum helps coordinate body movements. The caudate nucleus and the putamen receive the majority of input from cortical glutamatergic neurons as well as dopaminergic afferents of the substantia nigra, pars compacta, and

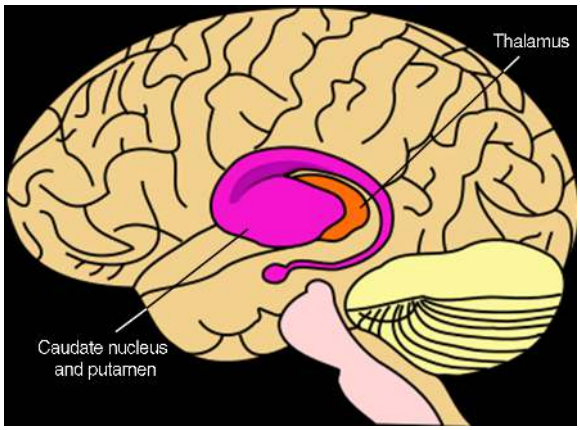



**Figure 40.1** Image showing the basal ganglia and related structures. The caudate nucleus and putamen and the thalamus are shown. *Source*: John Henkel, US Food and Drug Administration.

glutamatergic afferents of the thalamus [24]. Glutamate (glutamic acid) is abundant in the human body, but particularly in the nervous system, and is especially prominent in the human brain, where it is the body's most prominent neurotransmitter, the brain's main excitatory neurotransmitter, and also the precursor for γ-aminobutyric acid, the brain's main inhibitory neurotransmitter. Glutamate receptors are responsible for the glutamate-mediated postsynaptic excitation of neural cells and are important for neural communication, memory formation, learning, and regulation.

Analysis of several imaging studies has revealed four stages of neuropathology in HD. Beginning at stage 1 prior to the onset of symptoms, there appears to be selective cortical thinning [26] and alterations in the white matter of the corpus callosum [27]. Functional magnetic resonance imaging has revealed a decrease in the activity of the dorsolateral prefrontal cortex and reductions in grey-matter volume [28,29]. Autoradiographic techniques have also revealed a decrease in binding for cannabinoid, $D_2$ dopamine, and adenosine receptors, with a slight increase of γ-aminobutyric acid receptor binding [30]. An autoradiograph is an image on an X-ray film or nuclear emulsion produced by the pattern of decay emissions (e.g., β-particles or γ-rays) from a distribution of a radioactive substance. Alternatively, an autoradiograph may also be available as a digital image (digital autoradiography), due to the recent development of scintillation gas detectors or rare earth phosphorimaging systems.

At stage 2 of HD a selective degeneration of encephalin-containing striatal neurons projecting to the external segment of the globus pallidus is observed, as well as an increase in degeneration of substance-P-containing striatal neurons, projecting to the substantia nigra and pars reticulate [25]. Encephalins are related to the endorphins and have similar physiological effects.

At stage 3 of HD there is a progressive and severe loss of striatal projection neurons as well as thinning of cortical layers III, V, and VI.

Stage 4 is characterized by 95% loss of all striatal neurons and marked decreases in the size of the subthalamic nucleus, hypothalamus, white matter, and the cerebellum [31].

### 40.2.3 Mechanisms of neurodegeneration

Understanding the mechanism of degeneration in HD is difficult. The huntingtin protein HTT is ubiquitously expressed throughout the body, but is highly expressed in the central nervous system, particularly in corticostriatal projection neurons in layers III and V with projections to the striatum [32,33]. At the cellular level HTT is found in many subcellular compartments including the nucleus, dendrites, axons, and synapses of neurons, as well as in intracellular organelles [34,35]. Given its cellular diversity, the exact function of HTT remains unclear. However, the interactions of HTT with proteins involved in vesicle transport and cytoskeletal and synaptic structure suggest that HTT promotes cellular health and function.

The expanded CAG repeat near the N-terminus of *HTT*, leading to the formation of mutant HTT (mHTT), results in a gain-of-function disruption in a variety of cellular processes, including transcriptional dysregulation and transport of brain-derived neurotrophic factor (BDNF) [1,36], hypersensitivity of MSNs [37], and mitochondrial dysfunction [38].

Reductions of BDNF within the HD brain have been well documented [39,40]. Brain-derived neurotrophic factor is a member of the neurotrophin family of growth factors, which are related to the canonical nerve growth factor.

Produced in cortical neurons with afferent projections to the striatum, BDNF is transported via corticostriatal tracts to MSNs and is necessary for MSN survival [41,42]. Protein–protein interactions with mHTT occur at the stages of BDNF production, transport, release, and uptake [1]. It has been observed that symptomatic HD patients with lowered levels of BDNF had significantly worse motor and cognitive performances than individuals with normal BDNF levels, highlighting the importance of BDNF in the prevention of neuronal degeneration in HD [43,44].

Similarly, mHTT is involved in corticostriatal glutamatergic signaling. In particular, mHTT seems to impart a hypersensitivity of *N*-methyl-D-aspartate (NMDA) receptors to glutamate released from cortical projection neurons [45]. Coupled with reductions of mHTT-dependent reuptake of glutamate from the extracellular space [46], a cascade of events is initiated leading to the death of MSNs, termed excitotoxicity [37,47]. This increased excitability at the level of NMDA receptors creates an increase in the levels of intracellular calcium, leading to activation of proteases and lipases, generation of free radicals and constitutive nitric oxide synthase, disruption of mitochondrial oxidative phosphorylation, and enhanced brain lipid peroxidation, culminating in cell death by the release of apoptotic factors and oxidative stress [48,49].

The excitotoxicity hypothesis of HD is supported by evidence in which mHTT interacts with the surface of mitochondria to exhibit a defect in regulating intracellular calcium [50].

Mitochondria of HD patients also exhibit a defect in energy metabolism [38]. Additionally, reports indicate that glucose metabolism is altered beginning in the early stages of HD [51]. Molecular analysis of mitochondria of HD-affected individuals suggests dysfunction in complexes I, II, III, and IV of the electron transport chain that reduces oxidative phosphorylation, thereby reducing succinate oxidation and ATP synthesis [38,51,52].

## 40.3 Animal models

Several animal models of HD have been developed, many of which allow for the study of the behavioral deficits mimicking the progressive nature of HD. Animal models range from transgenic mice, in which a copy of *mHTT* is introduced into the animal's genome (Table 40.1), to chemical models, in which toxins are introduced to mimic the degeneration which occurs in HD (Table 40.2). These models have led to greater understanding of the molecular mechanisms of HD and have also allowed for the testing of potential therapies that are designed to slow the degeneration or, perhaps, replace MSNs lost during the progression of the disease.

### 40.3.1 Transgenic models

Amongst a myriad of mouse models in which a transgene with an expanded CAG has been inserted into the genome, or in which an insertion of CAG repeats on the endogenous *HTT* has been made, two mouse models are predominantly used in HD research: the R6/2 and YAC128 mice [1]. The R6/2 transgenic mouse expresses only the first exon of the human *HTT* gene with an expanded CAG repeat length of approximately 140–160 CAG repeats [53]. This model presents with a rapidly progressing phenotype in which robust motor deficits that include dyskinesia, irregular gait, and clasping are observed. The onset of the deficits appears between 6 and 8 weeks of age, with a lifespan of 13–15 weeks of age [59,60] in comparison with a normal murine lifespan of 2 years. However, aside from intranuclear inclusions of mHTT aggregates and striatal atrophy, many of the hallmark neuropathological symptoms of HD are not observed [1,53]. Unlike the R6/2 mouse, the YAC128 transgenic mouse expresses full-length *mHTT* with 128 CAG repeats [54,61]. These mice display a mild phenotype with forelimb and hindlimb coordination deficits appearing at 24 weeks of age [61], as well as robust striatal and cortical atrophy which includes a significantly reduced number of neurons and formation of mHTT aggregates within the striatum by 48 weeks of age [1,54].

Owing to technological and logistical issues, development of transgenic rat models of HD have lagged behind those of transgenic mouse models [62]. The tgHD (transgenic Huntington disease) 51CAG rat carries *mHTT* with 51 CAG repeats and exhibits a progressive phenotype resembling adult-onset HD [55]. These rats display early cognitive deficits [63] followed by motor dysfunction [55]. Similar to the R6/2 transgenic mouse model, the tgHD 51CAG rat displays minor neuropathological deficits with enlargement of the lateral ventricles as well as aggregation of mHTT [1,55].

**Table 40.1** Transgenic models of HD.

| | R6/2 mouse | YAC128 mouse | tgHD rat |
|---|---|---|---|
| Reference | [53] | [54] | [55] |
| CAG repeat | 140–160 | 128 | 51 |
| Onset | 5–6 weeks | 8–12 weeks | 40–50 weeks |
| Neuropathology | Atrophy of the striatum; aggregation of mHTT | Atrophy of the striatum and cell loss (48 weeks); aggregation of mHTT | Enlarged lateral ventricles; aggregation of mHTT |
| Motor deficits | 5–6 weeks | 24 weeks | 40–50 weeks |
| Weight loss | Yes | No | Yes |
| Lifespan | 13–15 weeks | Normal | 98 weeks |

**Table 40.2** Chemical models of HD.

| | Quinolinic acid | 3-Nitropropionic acid |
|---|---|---|
| Reference | [56] | [57,58] |
| Mechanism | NMDA receptor agonist | MSN mycotoxin |
| Administration | Single intrastriatal injection (unilateral or bilateral) | Progressive peripheral |
| Neuropathology | Immediate loss of MSNs | Progressive |
| Motor deficits | Immediate apomorphine/paw placement (unilateral lesions) and staircase test/rotarod (bilateral lesion) | Rotarod (2 weeks) |

## 40.4 *In vitro* models

Chemical models of HD were among the first to be used, and had a high degree of reproducibility that mimicked the neuropathology observed in HD (Table 40.2). Glutamate agonists such as kainic acid and quinolinic acid are able to selectively kill striatal MSNs through overactivation of NMDA receptors on the MSNs, which, in turn, causes lethal increases in intracellular calcium, a phenomenon that led to the development of the excitotoxic hypothesis of HD [32,56,64]. Single intrastriatal administration of quinolinic acid has shown immediate and selective degeneration of MSNs as well as motor and memory dysfunction, similar to what is observed in people with HD [65,66].

The mitochondrial toxin 3-nitropropionic acid (3-NP) was also an early chemical used to model HD [66–69]. Peripheral injections of 3-NP have been shown to cross the blood–brain barrier and irreversibly inhibit complexes II and III of the electron transport chain, inhibiting succinate dehydrogenase, and specifically reducing available energy within the striatum, similar to what is observed in HD [68,70,71]. Systematic intraperitoneal injections can replicate the progressive nature of HD, in which animals present with weight loss, motor dysfunction, and cell death [66,72].

## 40.5 Experimental therapies

Currently, no cure exists for HD, and only palliative treatments for reducing the motor symptoms of HD are available. The only therapy for HD approved by the US Food and Drug Administration to date is tetrabenazine, which acts to inhibit the hyperactive dopamine pathway, thereby reducing the severity of choreic movements [73,74]. Various pharmaceutical agents aimed at inhibiting the dopamine pathway, reducing excitotoxicity, or enhancing mitochondrial metabolism have shown promise in preclinical trials, but none has been able to progress beyond phase III clinical trials. Various gene therapies have also shown promise in preclinical settings. Among the more promising avenues for gene therapy is increasing expression of BDNF production from endogenous cells within the striatum via adenoviral or adeno-associated virus vector injections into the striatum [75–77]. These approaches were shown to be neuroprotective, with preservation of striatal MSNs following injection of quinalonic acid [76] and an increase in recruitment of neural stem cells to the striatum [75]. These methods also reduced motor deficits and prolonged lifespan in the transgenic R6/2 mouse model of HD [78,79].

Another target of gene therapy is to reduce transcription of *mHTT* through RNA interference. Most commonly, *HTT*-specific RNA interference has been introduced using viral vectors [80–82]. These studies were able to show nonspecific reduction of *HTT* expression in various mouse models of HD, as well as in healthy nonhuman primates [83]. A second method being explored is the use of antisense oligonucleotides that target common single nucleotide polymorphisms located on the *mHTT* allele. This method of gene therapy has been shown to specifically reduce mHTT expression in a humanized mouse model of HD (97 CAG/18 CAG) [84,85]. While these potential therapies hold promise, they lack the ability to replace cells that have been lost to the disease.

## 40.6 Cell transplantation

Transplantation of cells falls into two separate strategies: either to provide protection against neurodegeneration, or to replace the neurons lost because of the disease. Transplantation of mesenchymal stem/stromal cells (MSCs) has been traditionally used in the first (prophylactic) strategy, because of their ability to release neurotrophic factors that promote neuronal survival. Transplants of fetal or adult neural stem cells (NSCs) and the differentiated progeny of induced pluripotent stem cells (iPSCs) have been used in the second (replacement) strategy, since these cells can differentiate into neurons.

### 40.6.1 Mesenchymal stem/stromal cells

A true adult mesenchymal stem cell that fulfills the criteria of both self-renewal and lineage differentiation has been documented, but it is a rare cell, being present at a frequency of 1 in $10^4$ to 1 in $10^5$ in murine bone marrow. To get sufficient mesenchymal cells for laboratory experiments or for clinical trials, their progeny need to be expanded in *ex vivo* culture. These culture-expanded cells are known as mesenchymal stromal cells.

Transplantation of MSCs early in disease progression provides an option for intervention that can help protect against further degeneration. Originally described by Friedenstein *et al.* [86], MSCs are multipotent stromal cells that have since been defined by three main characteristics: (1) plastic adherence and proliferation in *ex vivo* tissue culture; (2) ability to differentiate into a diverse set of mesodermal lineages *in vitro*, and (3) expression of characteristic but not unique cell surface markers [87]. Mesenchymal stromal cells cannot dedifferentiate to mesenchymal stem cells. However, since mesenchymal stromal cells are pericytes, forming part of the blood vessel wall in all vascularized tissues, they can therefore be derived from many tissue sources, including bone marrow, umbilical cord blood, and adipose tissue. They can then expanded in *ex vivo* tissue culture. Other characteristics of human MSCs, defined by the International Society for Cellular Therapy, include positive expression of the cell surface proteins CD73, CD90, and CD105 and lack of expression of CD34, CD45, and major histocompatibility complex class II molecules [88].

The intrinsic characteristics of MSCs, such as immunomodulation and neurotrophic factor release, have made these cells a candidate for cell-based therapies in HD. The ability of MSCs to modulate the local immune environment comes from cell-to-cell contact and through the release of interleukins and cytokines that interact with a wide range of immune cells, which in turn help to protect transplanted MSCs from acute and prolonged immune responses and rejection [89–92]. MSCs secrete many neurotrophic factors, including BDNF, glial-derived neurotrophic factor (GDNF), and nerve growth factor (NGF) [93,94]. Recent advances in cell characterization techniques have allowed researchers to explore the secretome of MSCs with the aim of identifying all secreted molecules, which may provide insight into the mechanisms mediating the benefits of transplanted MSCs [95].

As MSCs can be isolated from various sources, it is important to determine if MSCs maintain similar characteristics regardless of their source. For example, MSCs isolated from the bone

marrow may express proteins implicated in neuroprotection at different levels relative to MSCs isolated from the umbilical cord. To test this hypothesis we isolated mRNA transcripts from these two MSC types and assayed them using reverse transcriptase polymerase chain reaction. Results from this study suggested that the expression of neurotrophic factors and anti-inflammatory cytokines are highly variable. Furthermore, it is apparent that the length of time MSCs are kept in culture will also alter expression of these genes and cell surface proteins (Figure 40.2). Given the degree of variability, it is unclear as to which MSC source and what the optimal duration of culture (number of passages) are that will produce the greatest benefit in a transplantation paradigm.

Early transplantation studies in animal models of HD focused on isolating MSCs derived from bone marrow, as these cells were particularly easy to isolate and expand in numbers sufficient for transplantation [94,96,97]. In 2003 we transplanted autologous whole bone marrow into the brains of rats treated with quinalonic acid [97]. Although reduction in working memory errors were observed, no evidence of neuronal differentiation of the transplanted cells was found, suggesting that the beneficial effects were the result of some mechanism other than cell replacement. We later observed that MSCs transplanted into the 3-NP rat model of HD had improved performance on the rotarod test, as well as a reduction in the size of the lesion in the striatum. We hypothesized that this was a result of release of neurotrophic

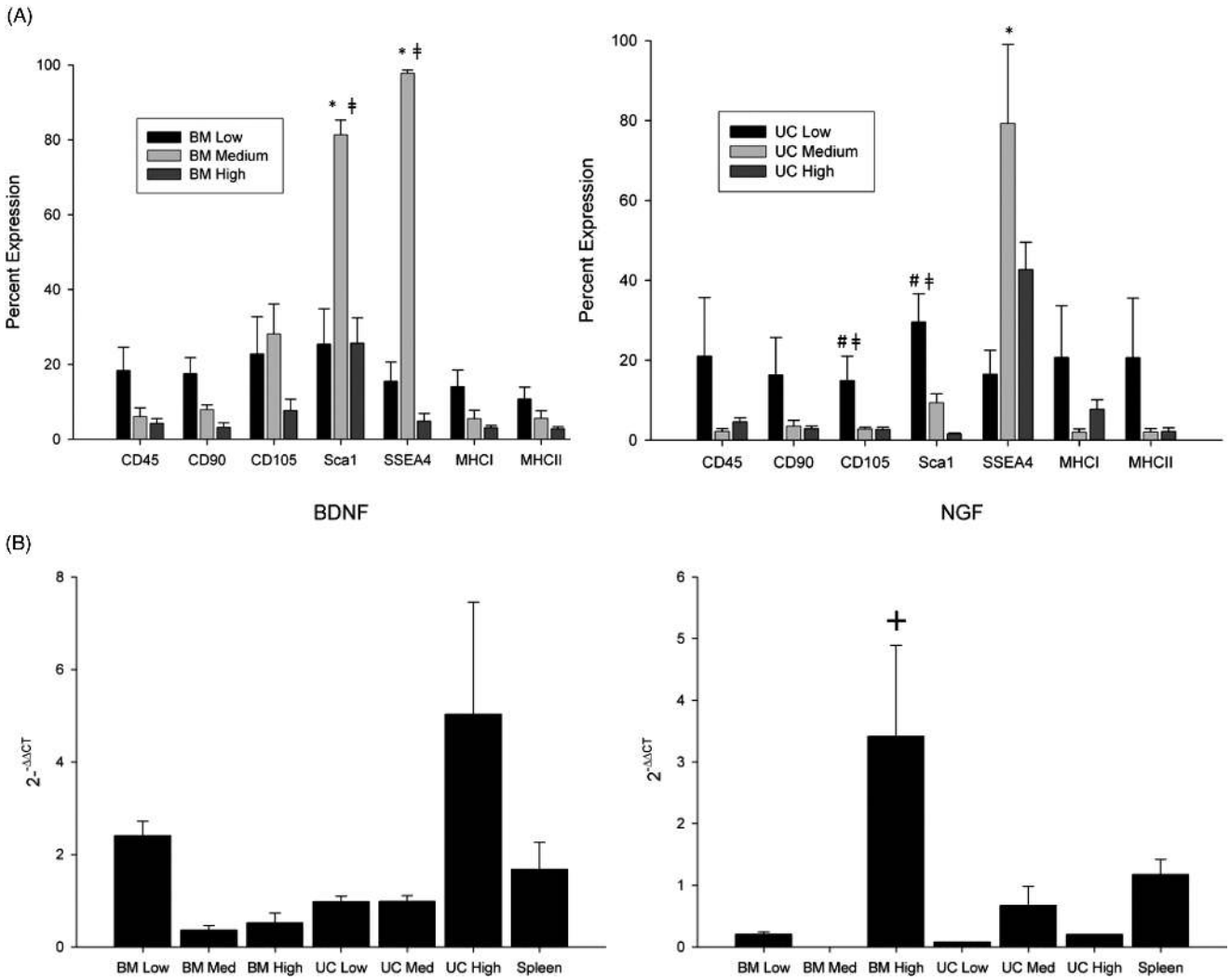



**Figure 40.2** (A) Expression of cell surface markers on MSCs derived from the bone marrow (BM) or umbilical cord (UC) over multiple passages and analyzed by flow cytometry. Changes in protein expression were observed across low passage (P) number (P4–P10), medium passage number (P40–P60), or high passage number (P100–P130). (B).Similarly, a high degree of variability was observed in mRNA transcripts for BDNF and NGF. These data indicate that different populations of MSCs exist, which may be able to be distinguished according to tissue sources or by duration in culture. Symbols: ∗, significantly different from low-passage cells; #, significantly different from medium-passage cells; ǂ, significantly different from high-passage cells; +, significantly different from all groups.

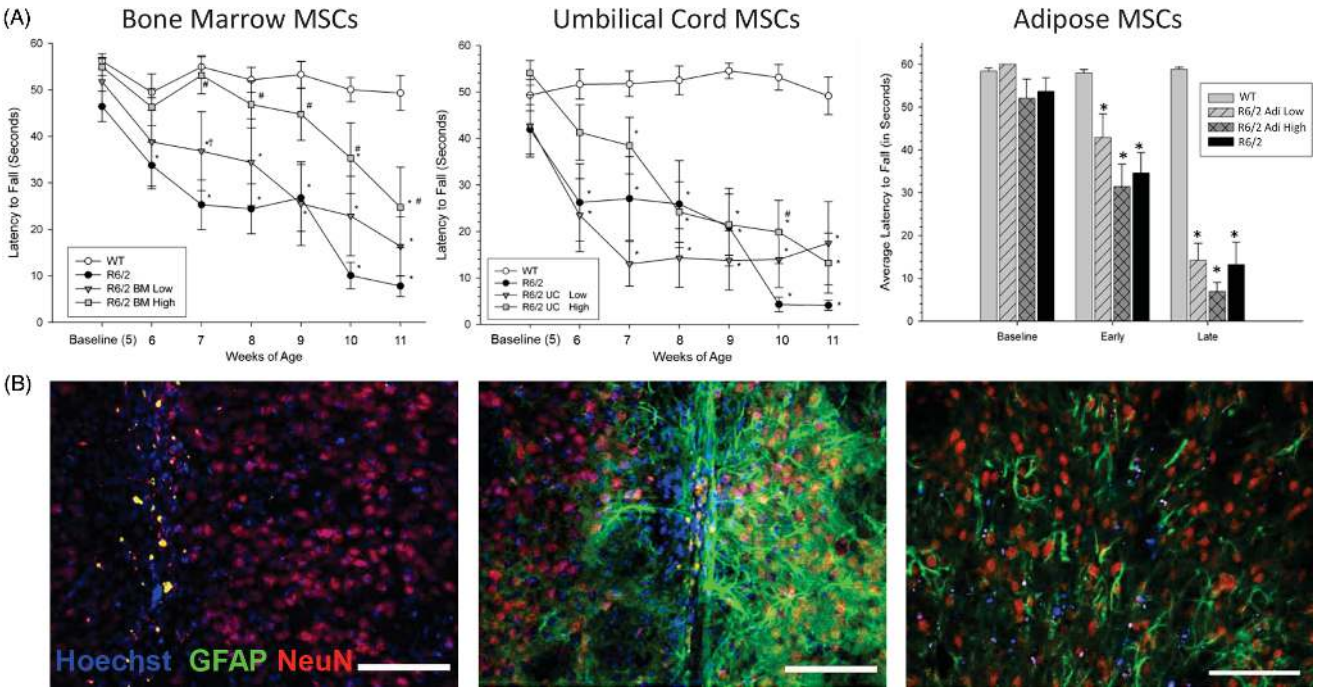



**Figure 40.3** Behavioral and histological evaluation following intrastriatal transplantation of low-passage (P4–10) or high-passage (P40–P60) MSCs derived from bone marrow (BM), umbilical cord (UC), and adipose tissue (Adi) in the R6/2 mouse model of HD. (A) It was observed that R6/2 mice transplanted with high-passage BM MSCs demonstrated greater behavioral sparing on the rotarod task, whereas R6/2 mice transplanted with UC MSCs or Adi MSCs did not display the same degree of benefit. (B) At 6.5 weeks following transplantation, Hoechst-labeled MSCs (blue) were observed at the site of transplantation in all groups. Activation of reactive astrocytes (green) was observed surrounding the site of transplantation in R6/2 mice transplanted with UC MSCs (center) and Adi MSCs (right) but not BM MSCs (left). There was no indication of neuronal differentiation of transplanted MSCs into neurons (red) in any of the three groups. (See color plate section.) Symbols: ∗, significantly different from wild type; #, significantly different from R6/2; †, significantly different from high passage. Scale bars represent 100 μm. *Source*: adapted from Fink 2013 [98], p. 130.

factors from the MSCs [94]. The rotarod test is a performance test based on a rotating rod with forced motor activity being applied, usually by a rodent. The test measures parameters such as riding time (seconds) or endurance. Some of the functions of the test include evaluating balance, grip strength, and motor coordination.

As mentioned above, MSCs have been derived from many tissues and organs, including the umbilical cord and adipose tissue, and these have provided additional MSC types for transplantation. As the R6/2 transgenic mouse model of HD presents with early symptoms and rapid progression, transplantation of MSCs derived from bone marrow, umbilical cord, and adipose tissue have provided high-throughput validation of the neuroprotective effects of these cells [98]. In three separate studies, results suggested that bone-marrow-derived MSCs may provide the greatest degree of behavioral and neuroanotomical sparing (Figure 40.3). Using immunohistochemistry, MSCs were detected in R6/2 mice transplanted with MSCs from bone marrow, umbilical cord, or adipose tissue for up to 6.5 weeks after transplantation. Furthermore, histological examination of the brains of R6/2 mice transplanted with bone-marrow-derived MSCs revealed a reduction in reactive astrocyte response. While the mechanisms behind the observed beneficial effects are still unclear, it has been suggested that neurotrophic factor support and immunomodulation provide a favorable microenvironment to sustain the function of endogenous MSNs [94].

### 40.6.2 Genetic engineering of mesenchymal stem/stromal cells

The direct correlation between intrastriatal levels of BDNF and disease progression led one group to alter cell culture conditions in order to induce MSCs to release enhanced amounts of BDNF and GDNF (Figure 40.4) [99,100]. When transplanted prior to quinolinic acid injection these neurotrophic-factor-releasing MSCs were able to reduce lesion size relative to nontransplanted controls [99]. This technique of enhancing neurotrophic factor release was also utilized in MSCs derived from human bone marrow and subsequently transplanted into R6/2 mice. This resulted in a transient reduction in rotarod deficits [100].

In an effort to overexpress neurotrophic factors in MSCs we used a retrovirus to transfect MSCs with the *BDNF* gene on a cytomegalovirus promoter, which led to the overexpression of BDNF [101]. Analysis of BDNF production in these cells revealed a sixfold increase in the amount of intracellular BDNF and a fourfold increase in secreted BDNF relative to production by nontransfected MSCs. These cells were then transplanted into

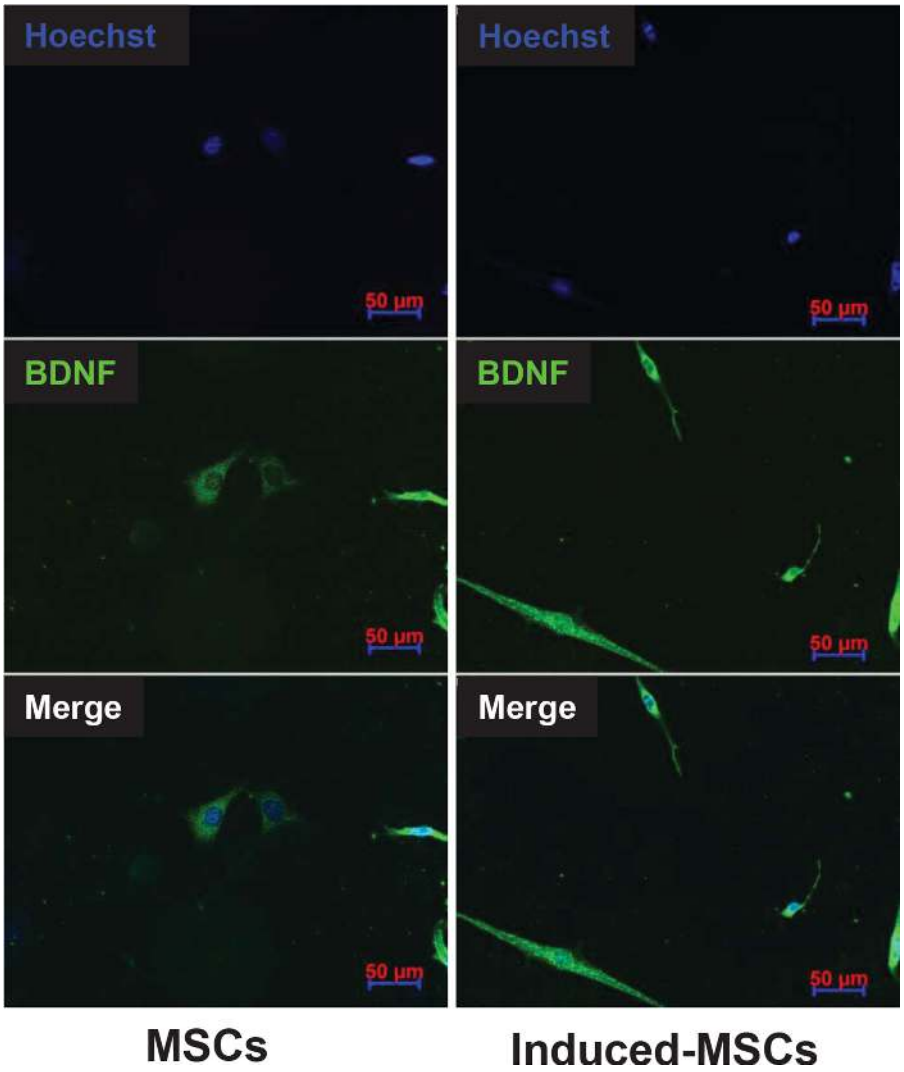



**Figure 40.4** Immunocytochemical analysis of intracellular BDNF of MSCs or MSCs induced to release neurotrophic factors (induced-MSCs). Following treatment with mitogens, basic fibroblast-derived growth factor, and epidermal growth factor, an increase in the fluorescent signal of immunolabeled BDNF (green) in induced-MSCs was observed relative to noninduced MSCs. (See color plate section.)

the YAC128 transgenic mouse model of HD and observed for 9 months. All YAC128 mice that were transplanted with the BDNF-engineered MSCs displayed an increased latency to fall from the rotarod and reduced clasping scores (Figure 40.5). Compared with R6/2 mice transplanted with unmodified MSCs, BDNF-expressing MSCs showed the highest amount of behavioral protection. Histological analysis revealed that YAC128 mice transplanted with BDNF-expressing MSCs had complete preservation of neurons in the striatum as well as protection against significant losses in the number of MSNs.

Viral transfection of MSCs has now progressed beyond the ability to increase levels of neurotrophic factors. Given that MSCs can establish cell-to-cell interactions to transfer mitochondria as well as RNA and other nucleic acids through extensions of the cytoplasm [102–105], Olson *et al.* measured the ability for short hairpin (sh)RNA produced by viral transfection in MSCs to reduce expression of HTT in neighboring cells [106]. Results from this *in vitro* study demonstrated that shRNA-expressing MSCs co-cultured with mHTT-expressing cells were able to reduce levels of the mutated mRNA within the cell as well as the expression of the mutated protein.

### 40.6.3 Embryonic and fetal stem cells

Pluripotent stem cells have garnered much attention in the study of neurodegenerative disorders primarily because of their potential to differentiate into any tissue of the three germ lineages: ectoderm (which includes neurons), endoderm, and mesoderm. Embryonic stem cells are derived from the inner cell mass of a blastocyst and express the transcription factors OCT4 and SOX2 [107,108]. Obtaining true pluripotent stem cells suitable for therapeutic transplantation has raised ethical concerns because the embryo is destroyed in the process.

Cells derived from fetal tissue have been utilized for transplantation with the hope that the fetal ganglionic eminence – cells

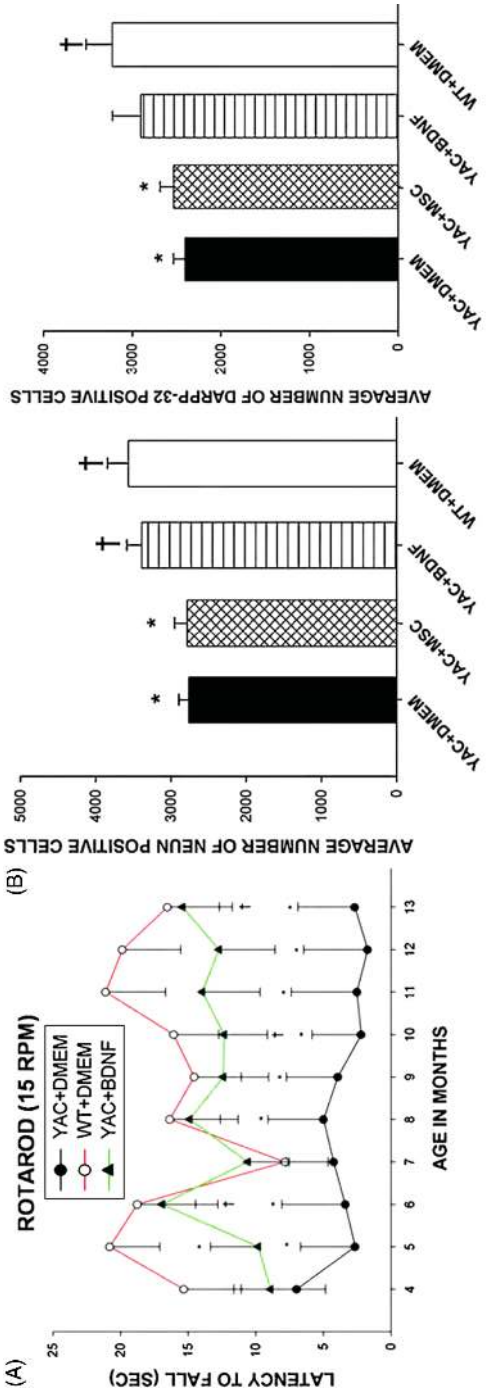



**Figure 40.5** Behavioral and histological evaluation following intrastriatal transplantation of MSCs genetically engineered to overexpress BDNF in the YAC128 mouse model of HD. (A) Following transplantation, a significant reduction of deficits in rotarod performance was observed in YAC128 mice transplanted with BDNF-MSCs relative to YAC128 mice receiving intrastriatal vehicle injections alone. (B). Relative to vehicle-injected YAC128 mice and YAC128 mice transplanted with unmodified MSCs, a preservation of striatal neurons (left) and MSNs (right) was observed in YAC128 mice transplanted with BDNF-MSCs. Symbols: *, significantly different from wild type; †, significantly different from YAC128 mice. DMEM: Dulbecco's modified Eagle's medium. *Source*: adapted from Dey 2010 [101], pp. 193–200. Reproduced with permission of Elsevier.

destined to become MSNs of the basal ganglia – will differentiate into MSNs following transplantation. Long-term clinical studies in which cells from the ganglionic eminence were transplanted into HD patients have shed light on the variability associated with fetal cell transplants. In 2000, Bachoud-Lévi and colleagues transplanted HD patients with ganglionic eminence tissue and found that three of the five patients receiving the transplants had a delay in the progression of their disease and that transplanted tissue was metabolically active up to 10 years after transplantation [109–111]. These beneficial effects, however, were not seen in all patients receiving transplantation of fetal ganglionic eminence tissue. Other groups observed short-term graft survival, HD-related neurodegeneration, and aggregate formation within the graft or formation of cysts at the site of transplantation [112–114]. These findings offer insight into clinical applications of fetal grafts, but the logistical challenges of their transplantation into HD patients provided the impetus to investigate other cell types suitable for such transplantation.

### 40.6.4 Neural stem cells

When large populations of proliferating cells were discovered in the adult subgranular zone of the hippocampus and subventricular zone or the lateral ventricles, the dogma that only a fixed number of cells exist in the brain was no longer tenable [115–117]. The extraction and expansion of NSCs has allowed researchers to obtain small quantities of tissue and produce large enough numbers of cells for *in vivo* transplantation. Culturing of NSCs *in vitro* requires defined growth medium supplemented with mitogens to maintain proliferation, at which point NSCs will begin to form free-floating multicellular neurospheres (Figure 40.6) [118]. Removal of growth mitogens from culture conditions induces NSCs to differentiate into cells within the neural lineage such as neurons, glial cells, and oligodendrocytes.

The potential for NSCs to form new neurons following transplantation into animal models of HD has garnered much attention. Transplantation of immortalized human, mouse, and rat NSCs have all shown considerable promise, with reductions in behavioral deficits being observed in several animal models of HD, including measures of apomorphine-induced rotation, beam walking, and latency to fall off the rotarod [119–126]. Several laboratories have observed that, following transplantation, NSCs maintain protein markers for immature neurons, as well as markers of mature neurons, astrocytes, and even MSNs [122,126]. However, Johann *et al.* found that the majority of the transplanted NSCs differentiated into astrocytes and not into mature neuronal phenotypes [127]. The ability of transplanted NSCs to evade rejection by the immune system is limited. While one study found few transplanted NSCs surviving up to 20 weeks posttransplant [128], another study observed that transplanted NSCs were rapidly rejected after 14 days in a quinolinic acid mouse model of HD and after 28 days in the R6/2 model of HD [127]. Although local immune suppression

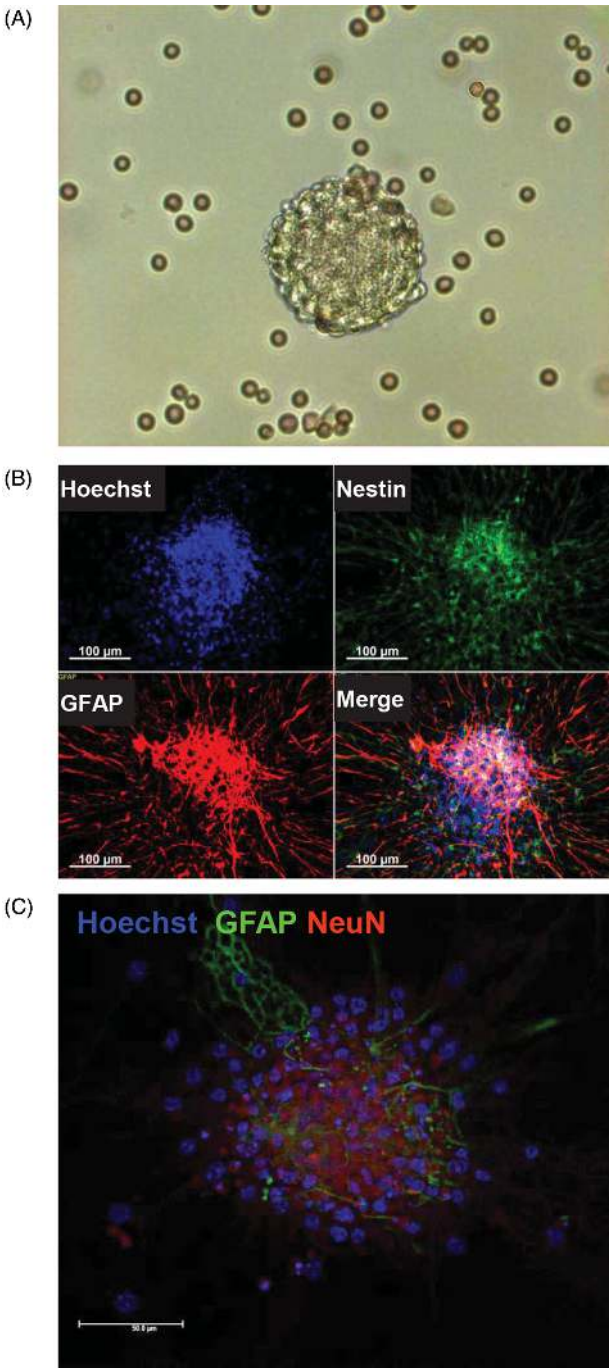



**Figure 40.6** Bright-field and fluorescent imaging of NSCs. (A) Phase-contrast image revealing that free-floating neurospheres are formed from cultured NSCs. (B). The Hoechst-labeled neurosphere (blue) is also labeled by the neural precursor marker nestin (green) and the astrocyte marker GFAP (red). (C). When cultured in the absence of growth mitogens the Hoechst-labeled neurosphere (blue) differentiates into astrocytes (green) and neurons (red). (See color plate section.)

can enhance graft survival, there are significant side effects associated with long-term immune suppression [129]. Identifying cells that can evade the host's immune response is of great value for cell-based transplantation approaches.

### 40.6.5 Induced pluripotent stem cells

In 2006, a team in Japan led by Shinya Yamanaka published a seminal paper showing that somatic cells could be reprogrammed by retroviral transfection of the genes for the embryonic transcription factors OCT3/4 and SOX2 together with the embryonic stem cell maintenance transcription factors KLF4 and c-MYC (commonly termed the "Yamanaka factors") [130]. These iPSCs exhibited many of the same characteristics of embryonic stem cells, including morphology, gene expression, and ability to differentiate into cells of all three germ lines. For HD, iPSCs have become a powerful research tool that has helped elucidate the mechanisms of mHTT at the single cell level, as well as providing a pluripotent cell that can be transplanted into the HD brain [131,132] (Figure 40.7).

Transplantation of neurally differentiated progeny of iPSCs into the central nervous system for therapeutic use remains a major topic of interest. Many practical hurdles remain, including the potential of transplanted iPSC progeny to develop the HD phenotype, as seen in fetal graft transplants [112], as well as their rejection by the recipient's immune system. Another major concern is the ability of undifferentiated iPSCs to form teratomas [133]. Controversy surrounds this issue, as some groups have observed teratomas [134–136] while other groups have not [132,137–139]. Techniques for reducing the tumorigenicity of iPSCs are being pursued and include lineage restriction toward NSCs or MSN progenitor cells prior to transplantation [133].

In 2012, Jeon *et al.* generated iPSCs from a patient with juvenile-onset HD (72 CAG repeats) and tested the potential of these cells to differentiate into MSNs *in vitro* and *in vivo* [140]. Following a standard *in vitro* multistage differentiation protocol, iPSCs were first differentiated into neural progenitors, which expressed markers for immature neural cells, as well as mature neural cells. When neural progenitor cells were transplanted into the quinolinic acid rat model and the YAC128 mouse model of HD [140,141] it was observed that the transplanted cells were able to express markers specific for MSNs as well as reducing behavioral deficits associated with these models. Interestingly, these HD-derived iPSCs did not show aggregates of mHTT, a hallmark of the HD brain.

The development of therapeutically relevant iPSCs for the treatment of HD is still in its infancy. We have established a line of iPSCs derived from healthy rat tail-tip-derived fibroblasts and transfected with a pair of nonintegrating adenoviruses expressing genes for the Yamanaka factors OCT4, KLF4, SOX2, and c-MYC [137]. The transfected fibroblasts formed iPSC colonies and expressed markers of pluripotency. To test the safety of adenovirus-generated iPSCs, undifferentiated iPSCs were bilaterally transplanted into the striata of immune-competent syngeneic rats. Transplanted iPSCs were observed up to 90 days posttransplant and expressed markers of mature region-specific neural cells. Formation of tumors was not observed.

To test the efficacy of adenovirus-generated iPSC transplantation in the HD brain we transplanted this line of cells into both

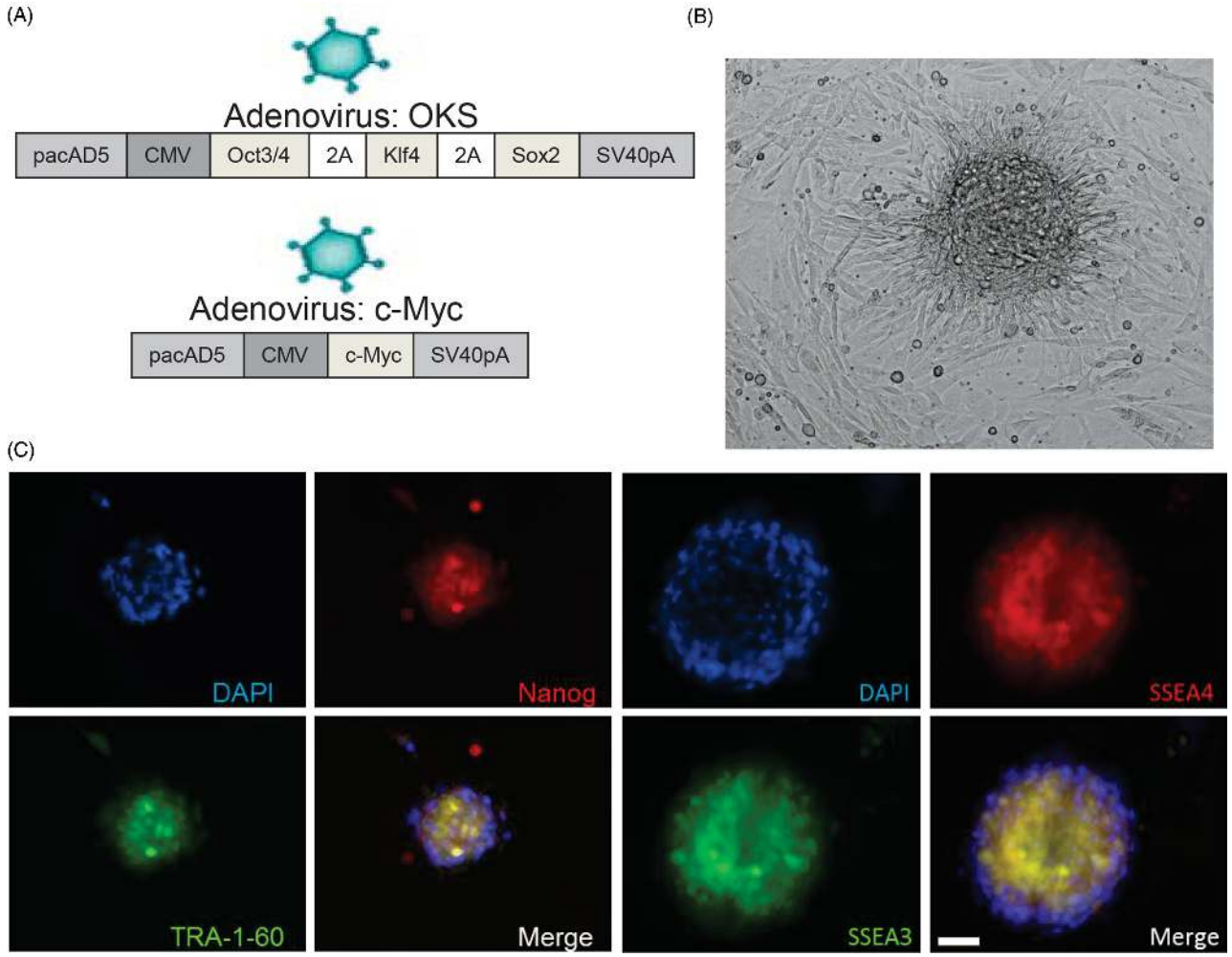



**Figure 40.7** Generation and characterization of iPSCs. (A). Schematic of the Yamanaka factors placed into two separate adenoviral vectors. The first adenoviral vector codes for Oct3/4, Klf4, and Sox2 and the second vector codes for c-Myc, each under the cytomegalovirus promoter. (B). When both adenoviral vectors are incorporated into rat tail-tip fibroblasts colonies are observed, which are then isolated and expanded on a monolayer of deactivated rat embryonic fibroblasts. (C). Immunocytochemical analysis of Hoechst-labeled iPSCs (blue) expressing pluripotency-related proteins, including Nanog, Tra-1-60, SSEA3, and SSEA4; scale bar: 50 µm. (See color plate section.) *Source*: adapted from Fink 2014 [132].

young and aged rats treated with 3-NP as well as the transgenic Huntington disease (tgHD) transgenic rat.

In the first study adenovirus-generated iPSCs were transplanted into syngeneic young adult (2 months of age) rats progressively dosed with 3-NP [132]. 3-NP is an inhibitor of the mitochondrial citric acid cycle and its administration results in a progressive locomotor deterioration resembling that of HD. In 3-NP rats that received transplants at stages mimicking early- and mid-stage HD, behavioral sparing on the accelerod test was observed (Figure 40.8). The accelerod test measures performance on the rod during acceleration at approximately 1 rev/s compared with that obtained using the rotarod when the same rod is operated at a constant speed of 20 rev/min. Surprisingly, a recovery of function was observed when 3-NP rats with end-stage disease were transplanted. Histological analysis of brains following transplantation showed survival of iPSCs with mature neuronal phenotypes as well as a reduction in neuronal loss due to 3-NP administration (Figure 40.9A).

However, in a second study aimed at better mimicking transplantation into adult-onset HD brain, the same line of iPSCs was transplanted into syngeneic older (6–8 months of age) rats treated with 3-NP. Six weeks after transplantation, histological analysis of these older 3-NP-treated rats given undifferentiated iPSCs revealed the presence of tumors and cystic lesions (Figure 40.9B). It is possible that an aged 3-NP rat model may not be an optimal choice for testing the efficacy of cell-based treatments, likely due to an enhanced inflammatory response to the 3-NP.

A third study was designed to determine whether iPSCs are effective in a transgenic rat model of HD. We transplanted the same line of iPSCs into allogeneic tgHD 51CAG rats. Results from this study indicated that survival of transplanted iPSCs was

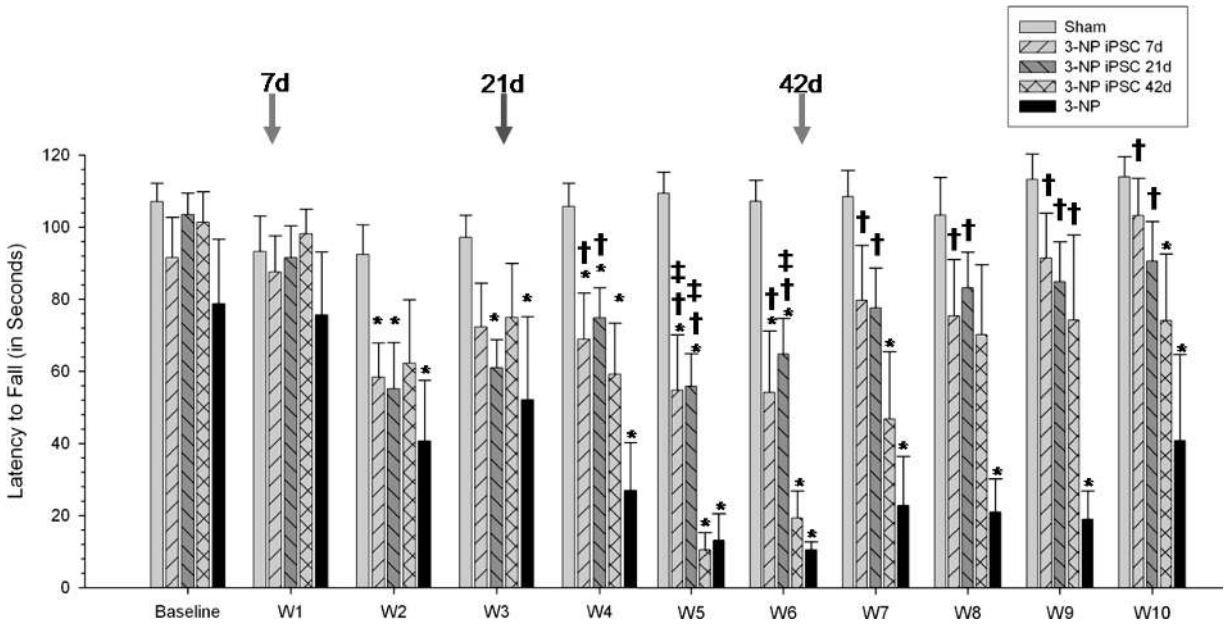



**Figure 40.8** Analysis of motor coordination in 3-NP-injected rats transplanted with undifferentiated iPSCs. Following a schedule of progressive 3-NP injections, rats were transplanted with undifferentiated iPSCs at 7, 21, and 42 days, representing early-, mid-, and late-stage HD. 3-NP rats transplanted at 7 and 21 days maintained motor coordination as observed by a significantly longer latency on the rotarod, relative to 3-NP rats injected with vehicle alone (black bars). Recovery of motor coordination deficits was observed in 3-NP rats transplanted with iPSCs 42 days after the start of the 3-NP injections. Symbols: ∗, significantly different from sham-treated control rats; †, significantly different from 3-NP-treated rats; ‡, significantly different from rats given 3-NP and iPSC 42 days previously. *Source*: adapted from Fink 2014 [132].

limited, with increased inflammation surrounding the transplant site (Figure 40.9C). However, no tumors were observed. Taken together, these studies suggest that the effectiveness of transplantation may be dependent upon age of the recipient, the genetic relatedness between iPSC donor and iPSC recipient (syngeneic versus allogeneic), and the choice of model.

Building on this, we then generated a similar line of iPSCs derived from healthy mouse tail-tip fibroblasts. Similar to

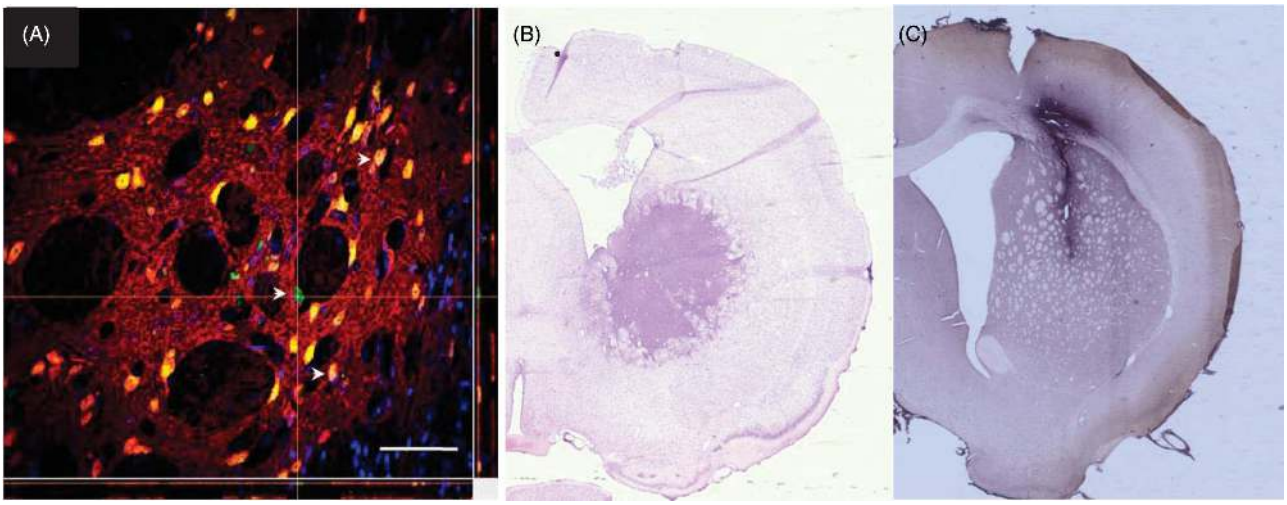



**Figure 40.9** Photomicrographs of tissue containing iPSCs that were transplanted into younger (2 months) and older (6–8 months) rats that had been treated with 3-NP as well as tgHD rats. (A). Immunohistochemical analysis of syngeneic transplantation of iPSCs in a young 3-NP-treated rat revealed that the Hoechst-prelabeled iPSCs (blue) survived and differentiated into mature neurons (green) or MSNs (yellow). (B). Following syngeneic transplantation of iPSCs into older 3-NP-treated rats, tumor-like masses were observed in the striatal tissue, which was stained with cresyl violet. (C). No prelabeled iPSCs were observed in the brains of tgHD rats 20 weeks after allogeneic transplantation of iPSCs, although a marked increase in macrophage activity (measured by IBA1 labeling) was observed at the site of transplantation. (See color plate section.)

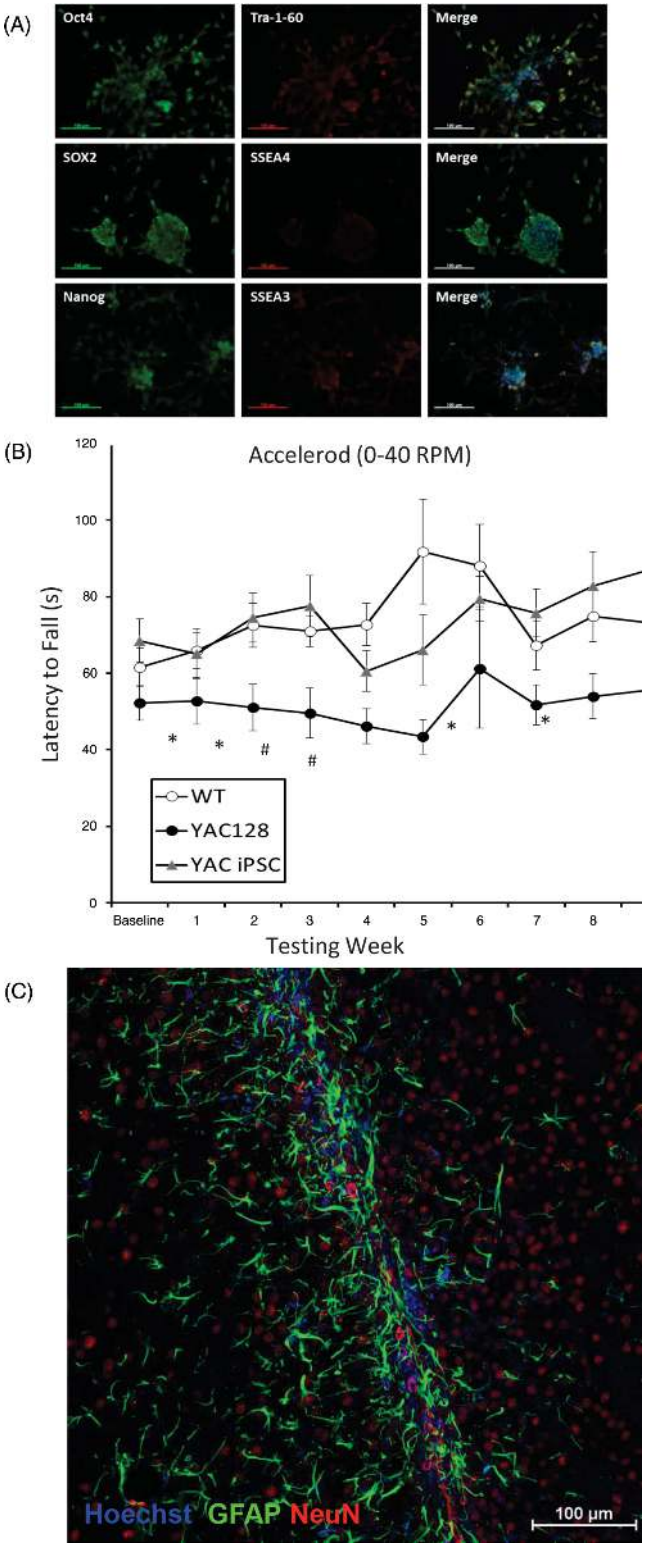



**Figure 40.10** Characterization and transplantation of adenovirus-generated iPSCs derived from mouse tail-tip fibroblasts. (A). Immunocytochemical analysis of Hoechst-labeled iPSCs (blue) shows that they express pluripotency proteins including OCT-4, SOX2, Nanog, Tra-1-60, SSEA3 m and SSEA4. (B). Accelerod analysis of YAC128 mice transplanted with iPSCs at 10.5 months of age revealed preservation of motor coordination for up to 10 weeks after transplantation. (C). Immunohistochemical analysis of iPSCs injected into the striatum of YAC128 mice showed the survival of Hoechst-prelabeled iPSCs (blue) and the differentiation of these cells into mature neurons (red) as well as astrocytes (green). (See color plate section.) Symbols: ∗, significantly different from all groups; #, significantly different from wild type mice.

rat-derived iPSCs, mouse-derived iPSCs express markers of pluripotency detectable by both flow cytometry and immunocytochemistry (Figure 40.10). When transplanted into syngeneic YAC128 mice at 12 months of age we observed a significant improvement in behavioral deficits and survival and differentiation of transplanted iPSCs up to 8 weeks following transplantation.

While the exact mechanisms for the observed effects of transplantation of iPSCs into the HD brain are unknown, it is encouraging that iPSCs can survive and differentiate into region-specific neurons in rodent models of HD. The recovery of function in late-stage 3-NP-treated rats, as well as improved function in aged transgenic mice, suggests that iPSCs are best suited toward transplantation when the HD brain is in a degenerated state.

### 40.6.6 Co-transplantation paradigm

To enhance the survival and differentiation of transplanted cells we have utilized the immunomodulatory and neurotrophic support of MSCs and co-transplanted them with NSCs into the tgHD rat model of HD [128]. It was observed that HD rats receiving transplants of both MSCs and NSCs had a greater degree of behavioral sparing as measured by the rotarod task relative to HD rats receiving transplants of NSCs alone. We also found that transplants of MSCs preserved the number of surviving NSCs, as determined by the larger graft size in the rats receiving the co-transplants (Figure 40.11). These findings suggest that the microenvironment provided by the co-transplanted MSCs allowed a greater survival of NSCs.

## 40.7 Conclusions

Although the genetic mutation underlying HD was identified more than 20 years ago, a unifying hypothesis to explain the mechanism for the selective degeneration of MSNs has yet to be elucidated. This has been a limiting factor in the development of targeted therapeutic agents for this disease. Presently, two treatment strategies for HD are being explored. The first strategy involves using cells prophylactically to slow the progression of the disease-induced deficits. With this approach the primary focus has been on the ability of transplanted MSCs to secrete an array of proteins that can alter the microenvironment and provide niche support for the local MSNs. Genetic engineering of MSCs to overexpress such proteins is in its early stages but has provided a proof of principle to serve as the basis for future work.

The second strategy focuses on replacing the cells already lost due to the disease. For this, the potential of fetal tissue, NSCs, and

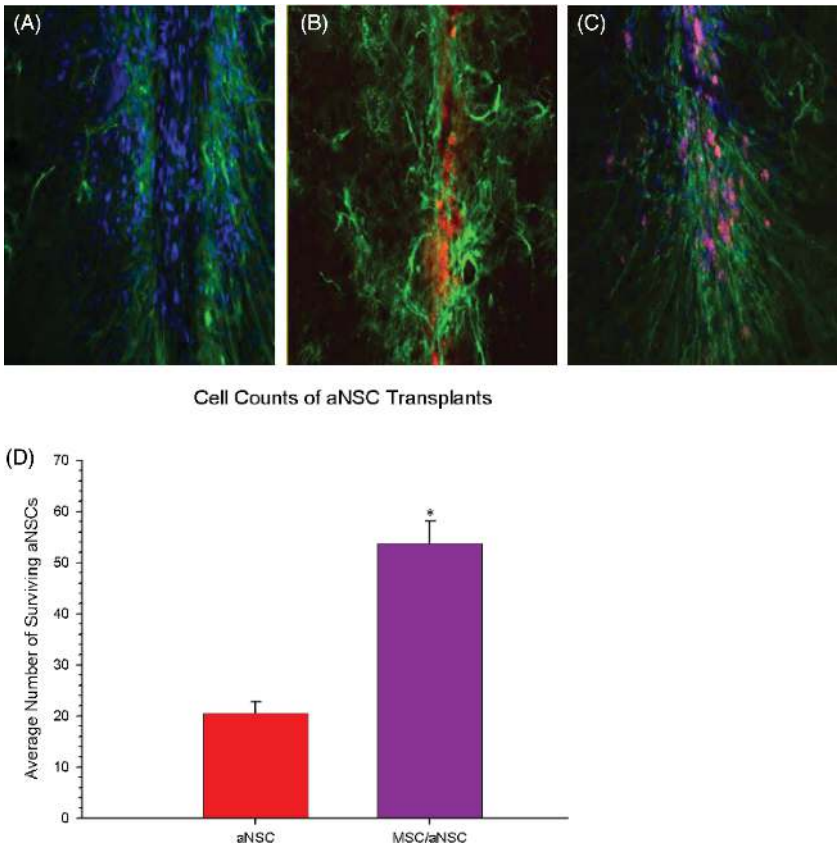



**Figure 40.11** Co-transplantation of adult NSCs (aNSCs) and MSCs in the tgHD rat model of HD. At 20 weeks after intrastriatal transplantation, (A) Hoechst-prelabeled MSCs (blue), (B) PKH-26-prelabeled aNSCs (red), or (C) co-transplanted MSCs and adult NSCs were observed. Reactive astrocytes (green) were also observed in all groups, although infiltration within the graft site was observed in NSC-transplanted animals; scale bar: 50 μm. (D). Rare event stereology at the site of transplantation demonstrated an increase in the number of positively labeled adult NSCs when co-transplanted with MSCs compared with tgHD rats transplanted with adult NSCs alone. Stereology is the three-dimensional interpretation of two-dimensional cross-sections of materials or tissues. (See color plate section.) Symbol: ∗, significantly different from adult NSCs. *Source*: adapted from Rossignol 2014 [128].

iPSCs has been harnessed, all of which have been shown capable of differentiating into MSNs. iPSCs offer an alternative to embryonic tissue but share the ability to differentiate into all cells of the body, including MSNs of the striatum. Results of early studies indicate that transplantation of iPSCs can reduce behavioral deficits and differentiate into mature neurons.

Combining the beneficial effects associated with each of these two strategies, such as co-transplantation of MSCs and NSCs, has provided another avenue for future therapeutic approaches. This could protect endogenous MSNs while enhancing the differentiation of transplanted NSCs or iPSCs to replace cells already lost. Understanding the characteristics of the various cell types, as well as determining how they exert their beneficial effects in animal models of HD, will provide valuable information toward ultimately using them clinically to improve both the longevity and quality of life for the individuals affected by this devastating disorder.

## References

1 C. Zuccato, M. Valenza, and E. Cattaneo. Molecular mechanisms and potential therapeutical targets in Huntington's disease. *Physiol. Rev.* 2010;**90**:905–981.

2 G. Huntington. On chorea. *Med. Surg. Report Phila.* 1872;**26**: 317–321.

3 R. Avila-Giron. Medical and social aspects of Huntington's chorea in the state of Zulia, Venezuela. *Adv. Neurol.* 1973;**1**:261–266.

4 J.F. Gusella, N.S. Wexler, P.M. Conneally, *et al.* A polymorphic DNA marker genetically linked to Huntington's disease. *Nature* 1983;**306**:234–238.

5 The Huntington's Disease Collaborative Research Group. A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. *Cell* 1993;**72**: 971–983.

6 S.C. Warby, R.K. Graham, and M.R. Hayden. Huntington disease [Internet] In: R.A. Pagon, M.P. Adam, H.H. Ardinger, *et al.*, editors. *GeneReviews®*. Seattle, WA: University of Washington, Seattle; 1993.

7 N.T. Potter, E.B. Spector, and T.W. Prior. Technical standards and guidelines for Huntington disease testing. *Genet. Med.* 2004; **6**:61–65.

8 D.R. Langbehn, R.R. Brinkman, D. Falush, *et al.* A new model for prediction of the age of onset and penetrance for Huntington's disease based on CAG length. *Clin. Genet.* 2004;**65**:267–277.

9 F.A. Nahhas, J. Garbern, K.M. Krajewski, *et al.* Juvenile onset Huntington disease resulting from a very large maternal expansion. *Am. J. Med. Genet. A* 2005;**137A**:328–331.

10 D.R. Langbehn, M.R. Hayden, J.S. Paulsen, and HD Investigators of the Huntington Study Group. CAG-repeat length and the age of

onset in Huntington disease (HD): a review and validation study of statistical approaches. *Am. J. Med. Genet. Part B Neuropsychiatr.* 2010;**153B**:397–408.
11 J.F. Gusella and M.E. MacDonald. Huntington's disease: the case for genetic modifiers. *Genome Med.* 2009;**1**:80.
12 N.A. Aziz, C.K. Jurgens, G.B. Landwehrmeyer, *et al.* Normal and mutant HTT interact to affect clinical severity and progression in Huntington disease. *Neurology* 2009;**73**:1280–1285.
13 A. Rosenblatt, K.-Y. Liang, H. Zhou, *et al.* The association of CAG repeat length with clinical progression in Huntington disease. *Neurology* 2006;**66**:1016–1020.
14 T. Pringsheim, K. Wiltshire, L. Day, *et al.* The incidence and prevalence of Huntington's disease: a systematic review and meta-analysis. *Mov. Disord.* 2012;**27** (9):1083–1091.
15 G. Bates, S. Tabrizi, and L. Jones (eds). *Huntington's Disease*. Oxford University Press: New York, 2014.
16 P. Gonzalez-Alegre and A.K. Afifi. Clinical characteristics of childhood-onset (juvenile) Huntington disease: report of 12 patients and review of the literature. *J. Child Neurol.* 2006;**21**:223–229.
17 T. Foroud, J. Gray, and J. Ivashina. Differences in duration of Huntington's disease based on age at onset. *J. Neurol. Neurosurg. Psychiatry* 1999;**66**:52–56.
18 J.S. Paulsen, D.R. Langbehn, J.C. Stout, *et al.* Detection of Huntington's disease decades before diagnosis: the Predict-HD study. *J. Neurol. Neurosurg. Psychiatry* 2008;**79**:874–880.
19 J.S. Paulsen, M. Hayden, J.C. Stout, *et al.* Preparing for preventive clinical trials: the Predict-HD study. *Arch. Neurol.* 2006;**63**:883–890.
20 V. Hahn-Barma, B. Deweer, A. Durr, *et al.* Are cognitive changes the first symptoms of Huntington's disease? *A study of gene carriers. J. Neurol. Neurosurg. Psychiatry* 1998;**64**:172–177.
21 J.S. Paulsen, C. Nehl, K.F. Hoth, *et al.* Depression and stages of Huntington's disease. *J. Neuropsychiatry Clin. Neurosci.* 2005;**17**: 496–502.
22 S.C. Kirkwood, J.L. Su, P. Conneally, *et al.* Progression of symptoms in the early and middle stages of Huntington disease. *Arch. Neurol.* 2001;**58**:273–278.
23 F.O. Walker. Huntington's disease. *Lancet* 2007;**369**:218–228.
24 I.J. Mitchell, A.J. Cooper, and M.R. Griffiths. The selective vulnerability of striatopallidal neurons. *Prog. Neurobiol.* 1999;**59**:691–719.
25 A. Reiner, R.L. Albin, K.D. Anderson, *et al.* Differential loss of striatal projection neurons in Huntington disease. *Proc. Natl. Acad. Sci. U. S. A.* 1988;**85**:5733–5737.
26 H.D. Rosas, N.D. Hevelone, A.K. Zaleta, *et al.* Regional cortical thinning in preclinical Huntington disease and its relationship to cognition. *Neurology* 2005;**65**:745–747.
27 H.D. Rosas, D.S. Tuch, N.D. Hevelone, *et al.* Diffusion tensor imaging in presymptomatic and early Huntington's disease: selective white matter pathology and its relationship to clinical measures. *Mov. Disord.* 2006;**21**:1317–1325.
28 R.C. Wolf, F. Sambataro, N. Vasic, *et al.* Longitudinal functional magnetic resonance imaging of cognition in preclinical Huntington's disease. *Exp. Neurol.* 2011;**231**:214–222.
29 R.C. Wolf, N. Vasic, C. Schönfeldt-Lecuona, *et al.* Dorsolateral prefrontal cortex dysfunction in presymptomatic Huntington's disease: evidence from event-related fMRI. *Brain J. Neurol.* 2007;**130**:2845–2857.
30 M. Glass, M. Dragunow, and R.L. Faull. The pattern of neurodegeneration in Huntington's disease: a comparative study of cannabinoid, dopamine, adenosine and $GABA_A$ receptor alterations in the human basal ganglia in Huntington's disease. *Neuroscience* 2000;**97**:505–519.
31 J.P. Vonsattel, R.H. Myers, T.J. Stevens, *et al.* Neuropathological classification of Huntington's disease. *J. Neuropathol. Exp. Neurol.* 1985;**44**:559–577.
32 A.M. Estrada Sánchez, J. Mejía-Toiber, and L. Massieu. Excitotoxic neuronal death and the pathogenesis of Huntington's disease. *Arch. Med. Res.* 2008;**39**:265–276.
33 F.R. Fusco, Q. Chen, W.J. Lamoreaux, *et al.* Cellular localization of huntingtin in striatal and cortical neurons in rats: lack of correlation with neuronal vulnerability in Huntington's disease. *J. Neurosci.* 1999;**19**:1189–1202.
34 P. Harjes and E.E. Wanker. The hunt for huntingtin function: interaction partners tell many different stories. *Trends Biochem. Sci.* 2003;**28**:425–433.
35 C. Landles and G. P. Bates. Huntingtin and the molecular pathogenesis of Huntington's disease. *EMBO Rep.* 2004;**5**:958–263.
36 F. Borovecki, L. Lovrecic, J. Zhou, *et al.* Genome-wide expression profiling of human blood reveals biomarkers for Huntington's disease. *Proc. Natl. Acad. Sci. U. S. A.* 2005;**102**:11023–11028.
37 M.M.Y. Fan and L.A. Raymond. *N*-Methyl-D-aspartate (NMDA) receptor function and excitotoxicity in Huntington's disease. *Prog. Neurobiol.* 2007;**81**:272–293.
38 I. Túnez, I. Tasset, V. Pérez-De La Cruz, *et al.* 3-Nitropropionic acid as a tool to study the mechanisms involved in Huntington's disease: past, present and future. *Mol. Basel Switz.* 2010;**15**:878–916.
39 C. Zuccato, A. Ciammola, D. Rigamonti, *et al.* Loss of huntingtin-mediated BDNF gene transcription in Huntington's disease. *Science* 2001;**293**:493–498.
40 C. Zuccato, M. Marullo, P. Conforti, *et al.* Systematic assessment of BDNF and its receptor levels in human cortices affected by Huntington's disease. *Brain Pathol. Zurich Switz.* 2008;**18**:225–238.
41 C.A. Altar, N. Cai, T. Bliven, *et al.* Anterograde transport of brain-derived neurotrophic factor and its role in the brain. *Nature* 1997;**389**:856–860.
42 S. Ivkovic, O. Polonskaia, I. Fariñas, and M.E. Erlich. Brain-derived neurotrophic factor regulates maturation of the DARPP-32 phenotype in striatal medium spiny neurons: studies *in vivo* and *in vitro*. *Neuroscience* 1997;**79**:509–516.
43 A. Ciammola, J. Sassone, M. Cannella, *et al.* Low brain-derived neurotrophic factor (BDNF) levels in serum of Huntington's disease patients. *Am. J. Med. Genet. B Neuropsychiatr. Genet.* 2007;**144B**:574–577.
44 P. Conforti, C. Ramos, B.L. Apostol, *et al.* Blood level of brain-derived neurotrophic factor mRNA is progressively reduced in rodent models of Huntington's disease: restoration by the neuroprotective compound CEP-1347. *Mol. Cell. Neurosci.* 2008;**39**:1–7.
45 Y. Sun, A. Savanenin, P.H. Reddy, and Y.F. Liu. Polyglutamine-expanded huntingtin promotes sensitization of *N*-methyl-D-aspartate receptors via post-synaptic density 95. *J. Biol. Chem.* 2001;**276**:24713–24718.
46 A.V. Tzingounis and J.I. Wadiche. Glutamate transporters: confining runaway excitation by shaping synaptic transmission. *Nat. Rev. Neurosci.* 2007;**8**:935–947.
47 R. Schwarcz, J.P. Bennett Jr. and J.T. Coyle. Inhibitors of GABA metabolism: implications for Huntington's disease. *Ann. Neurol.* 1977;**2**:299–303.

48 H.B. Fernandes and L.A. Raymond. NMDA receptors and Huntington's disease [Internet] In: A.M. Van Dongen (ed.), *Biology of the NMDA Receptor*. Boca Raton, FL: CRC Press, 2009.

49 H. Kalonia, P. Kumar, B. Nehru, and A. Kumar. Neuroprotective effect of MK-801 against intra-striatal quinolinic acid induced behavioral, oxidative stress and cellular alterations in rats. *Indian J. Exp. Biol.* 2009;**47**:880–892.

50 A.V. Panov, C.-A. Gutekunst, B.R. Leavitt, *et al*. Early mitochondrial calcium defects in Huntington's disease are a direct effect of polyglutamines. *Nat. Neurosci.* 2002;**5**:731–736.

51 S.E. Browne. Mitochondria and Huntington's disease pathogenesis. *Ann. N. Y. Acad. Sci.* 2008;**1147**:358–382.

52 B.G. Jenkins, W.J. Koroshetz, M.F. Beal, and B.R. Rosen. Evidence for impairment of energy metabolism *in vivo* in Huntington's disease using localized $^{1}$H NMR spectroscopy. *Neurology* 1993;**43**: 2689–2689.

53 L. Mangiarini, K. Sathasivam, M. Seller, *et al*. Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* 1996;**87**:493–506.

54 E.J. Slow, J. van Raamsdonk, D. Rogers, *et al*. Selective striatal neuronal loss in a YAC128 mouse model of Huntington disease. *Hum. Mol. Genet.* 2003;**12**:1555–1567.

55 S. von Hörsten, I. Schmitt, H.P. Nguyen, *et al*. Transgenic rat model of Huntington's disease. *Hum. Mol. Genet.* 2003;**12**:617–624.

56 M.F. Beal, N.W. Kowall, D.W. Ellison, *et al*. Replication of the neurochemical characteristics of Huntington's disease by quinolinic acid. *Nature* 1986;**321**:168–171.

57 E. Brouillet, B.G. Jenkins, B.T. Hyman, *et al*. Age-dependent vulnerability of the striatum to the mitochondrial toxin 3-nitropropionic acid. *J. Neurochem.* 1993;**60**:356–359.

58 D. Shear, K. Haik, and G. Dunbar. Creatine reduces 3-nitropropionic-acid-induced cognitive and motor abnormalities in rats. *Neuroreport* 2000;**11**:1833–1837.

59 Z. DeMarch, C. Giampà, S. Patassini, *et al*. Beneficial effects of rolipram in the R6/2 mouse model of Huntington's disease. *Neurobiol. Dis.* 2008;**30**:375–387.

60 K.P. Murphy, R.J. Carter, L.A. Lione, *et al*. Abnormal synaptic plasticity and impaired spatial cognition in mice transgenic for exon 1 of the human Huntington's disease mutation. *J. Neurosci.* 2000;**20**:5115–5123.

61 E.J. Slow, R.K. Graham, A.P. Osmand, *et al*. Absence of behavioral abnormalities and neurodegeneration *in vivo* despite widespread neuronal huntingtin inclusions. *Proc. Natl. Acad. Sci. U. S. A.* 2005;**102**:11402–11407.

62 O. Bugos, M. Bhide, and N. Zilka. Beyond the rat models of human neurodegenerative disorders. *Cell. Mol. Neurobiol.* 2009;**29**: 859–869.

63 K.D. Fink, J. Rossignol, A.T. Crane, *et al*. Early cognitive dysfunction in the HD 51 CAG transgenic rat model of Huntington's disease. *Behav. Neurosci.* 2012;**126**:479–487.

64 E.G. McGeer and P.L. McGeer. Duplication of biochemical changes of Huntington's chorea by intrastriatal injections of glutamic and kainic acids. *Nature* 1976;**263**:517–519.

65 D. Shear, J. Dong, K.L. Haik-Creguer, *et al*. Chronic administration of quinolinic acid in the rat striatum causes spatial learning deficits in a radial arm water maze task. *Exp. Neurol.* 1998;**150**:305–311.

66 D. Shear, J. Dong, C. Gundy, *et al*. Comparison of intrastriatal injections of quinolinic acid and 3-nitropropionic acid for use in animal models of Huntington's disease. *Prog. Neuropsychopharmacol. Biol. Psychiatry* 1998;**22**:1217–1240.

67 M.F. Beal, E. Brouillet, B.G. Jenkins, *et al*. Neurochemical and histologic characterization of striatal excitotoxic lesions produced by the mitochondrial toxin 3-nitropropionic acid. *J. Neurosci.* 1993;**13**:4181–4192.

68 E. Brouillet, F. Condé, M.F. Beal, and P. Hantrave., Replicating Huntington's disease phenotype in experimental animals. *Prog. Neurobiol.* 1999;**59**:427–468.

69 A.C. Ludolph, F. He, P.S. Spencer, *et al*. 3-Nitropropionic acid - exogenous animal neurotoxin and possible human striatal toxin. *Can. J. Neurol. Sci. J. Can. Sci. Neurol.* 1991;**18**:492–498.

70 P. Kumar, and A. Kumar. Neuroprotective effect of cyclosporine and FK506 against 3-nitropropionic acid induced cognitive dysfunction and glutathione redox in rat: possible role of nitric oxide. *Neurosci. Res.* 2009;**63**:302–314.

71 A. Lukács, A. Szabó, A. Papp, and T. Vezér. Altered open field behavior in rats induced by acute administration of 3-nitropropionic acid: possible glutamatergic and dopaminergic involvement. *Acta Biol. Hung.* 2009;**60**:359–367.

72 N. El Massioui, S. Ouary, F. Chéruel, *et al*. Perseverative behavior underlying attentional set-shifting deficits in rats chronically treated with the neurotoxin 3-nitropropionic acid. *Exp. Neurol.* 2001;**172**:172–181.

73 S. Frank, W. Ondo, S. Fahn, *et al*. a study of chorea after tetrabenazine withdrawal in patients with Huntington disease. *Clin. Neuropharmacol.* 2008;**31**:127–133.

74 Huntington Study Group. Tetrabenazine as antichorea therapy in Huntington disease: a randomized controlled trial. *Neurology* 2006;**66**:366–372.

75 E. Chmielnicki, A. Benraiss, A.N. Economides, and S.A. Goldman. Adenovirally expressed noggin and brain-derived neurotrophic factor cooperate to induce new medium spiny neurons from resident progenitor cells in the adult striatal ventricular zone. *J. Neurosci.* 2004;**24**:2133–2142.

76 A.P. Kells, D.M. Fong, M. Dragunow, *et al*. AAV-mediated gene delivery of BDNF or GDNF is neuroprotective in a model of Huntington disease. *Mol. Ther. J. Am. Soc. Gene Ther.* 2004;**9**: 682–688.

77 A.P. Bemelmans, P. Horellou, L. Pradier, *et al*. Brain-derived neurotrophic factor-mediated protection of striatal neurons in an excitotoxic rat model of Huntington's disease, as demonstrated by adenoviral gene transfer. *Hum. Gene Ther.* 1999;**10**:2987–2997.

78 A. Benraiss, M.J. Toner, Q. Xu, *et al*. Sustained mobilization of endogenous neural progenitors delays disease progression in a transgenic model of Huntington's disease. *Cell Stem Cell* 2013;**12**: 787–799.

79 S.-R. Cho, A. Benraiss, E. Chmielnicki, *et al*. Induction of neostriatal neurogenesis slows disease progression in a transgenic murine model of Huntington disease. *J. Clin. Invest.* 2007;**117**:2889–2902.

80 M. DiFiglia, M. Sena-Esteves, K. Chase, *et al*. Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proc. Natl. Acad. Sci. U. S. A.* 2007;**104**:17204–17209.

81 S. Harper, P.D. Staber, X. He, *et al*. RNA interference improves motor and neuropathological abnormalities in a Huntington's disease mouse model. *Proc. Natl. Acad. Sci. U. S. A.* 2005;**102**: 5820–5825.

82 E. Rodriguez-Lebron, E.M. Denovan-Wright, K. Nash, *et al.* Intrastriatal rAAV-mediated delivery of anti-huntingtin shRNAs induces partial reversal of disease progression in R6/1 Huntington's disease transgenic mice. *Mol. Ther. J. Am. Soc. Gene Ther.* 2005;**12**:618–633.

83 J.L. McBride, M.R. Pitzer, R.L. Boudreau, *et al.* Preclinical safety of RNAi-mediated HTT suppression in the rhesus macaque as a potential therapy for Huntington's disease. *Mol. Ther. J. Am. Soc. Gene Ther.* 2011;**19**:2152–2162.

84 M.E. Østergaard, A.L. Southwell, H. Kordasiewicz, *et al.* Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant huntingtin in the CNS. *Nucleic Acids Res.* 2013;**41**:9634–9650.

85 A.L. Southwell, N.H. Skotte, H.B. Kordasiewicz, *et al. In vivo* evaluation of candidate allele-specific mutant huntingtin gene silencing antisense oligonucleotides. *Mol. Ther.* 2014;**22**:2093–2106.

86 A.J. Friedenstein, J.F. Gorskaja, and N.N. Kulagina. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 1976;**4**:267–274.

87 N. Ahmadbeigi, M. Soleimani, Y. Gheisari, *et al.* Dormant phase and multinuclear cells: two key phenomena in early culture of murine bone marrow mesenchymal stem cells. *Stem Cells Dev.* 2011;**20**:1337–1347.

88 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

89 A.U. Engela, C.C. Baan, F.J.M.F. Dor, *et al.* On the interactions between mesenchymal stem cells and regulatory T cells for immunomodulation in transplantation. *Front. Immunol.* 2012;**3**:126.

90 M.D. Griffin, T. Ritter, and B. P. Mahon. Immunological aspects of allogeneic mesenchymal stem cell therapies. *Hum. Gene Ther.* 2010;**21**:1641–1655.

91 J. Rossignol, C. Boyer, R. Thinard, *et al.* Mesenchymal stem cells induce a weak immune response in the rat striatum after allo or xenotransplantation. *J. Cell. Mol. Med.* 2009;**13**:2547–2558.

92 Y. Shi, J. Su, A.I. Roberts, *et al.* How mesenchymal stem cells interact with tissue immune responses. *Trends Immunol.* 2012;**33**:136–143.

93 L. Crigler, R.C. Robey, A. Asawachaicharn, *et al.* Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Exp. Neurol.* 2006;**198**:54–64.

94 J. Rossignol, C. Boyer, X. Lévèque, *et al.* Mesenchymal stem cell transplantation and DMEM administration in a 3NP rat model of Huntington's disease: morphological and behavioral outcomes. *Behav. Brain Res.* 2011;**217**:369–378.

95 D. Drago, C. Cossetti, N. Iraci, *et al.* The stem cell secretome and its role in brain repair. *Biochimie* 2013;**95**:2271–2285.

96 K. Bantubungi, D. Blum, L. Cuvelier, *et al.* Stem cell factor and mesenchymal and neural stem cell transplantation in a rat model of Huntington's disease. *Mol. Cell. Neurosci.* 2008;**37**:454–470.

97 L. Lescaudron, D. Unni, and G.L. Dunbar. Autologous adult bone marrow stem cell transplantation in an animal model of Huntington's disease: behavioral and morphological outcomes. *Int. J. Neurosci.* 2003;**113**:945–956.

98 K.D. Fink, J. Rossignol, A.T. Crane, *et al.* Transplantation of umbilical cord-derived mesenchymal stem cells into the striata of R6/2 mice: behavioral and neuropathological analysis. *Stem Cell Res. Ther.* 2013;**4**:130.

99 O. Sadan, E. Melamed, and D. Offen. Intrastriatal transplantation of neurotrophic factor-secreting human mesenchymal stem cells improves motor function and extends survival in R6/2 transgenic mouse model for Huntington's disease. *PLoS Curr.* 2012;**4**: e4f7f6dc013d4e.

100 O. Sadan, N. Shemesh, R. Barzilay, *et al.* Mesenchymal stem cells induced to secrete neurotrophic factors attenuate quinolinic acid toxicity: a potential therapy for Huntington's disease. *Exp. Neurol.* 2012;**234**:417–427.

101 N.D. Dey, M.C. Bombard, B.P. Roland, *et al.* Genetically engineered mesenchymal stem cells reduce behavioral deficits in the YAC 128 mouse model of Huntington's disease. *Behav. Brain Res.* 2010;**214**:193–200.

102 M. Simons and G. Raposo. Exosomes - vesicular carriers for intercellular communication. *Curr. Opin. Cell Biol.* 2009;**21**:575–581.

103 H.H. Gerdes and R.N. Carvalho. Intercellular transfer mediated by tunneling nanotubes. *Curr. Opin. Cell Biol.* 2008;**20**:470–475.

104 P.B. Gahan and M. Stroun. The virtosome – a novel cytosolic informative entity and intercellular messenger. *Cell Biochem. Funct.* 2010;**28**:529–538.

105 J.L. Spees, S.D. Olson, M.J. Whitney, and D.J. Pockop. Mitochondrial transfer between cells can rescue aerobic respiration. *Proc. Natl. Acad. Sci. U. S. A.* 2006;**103**:1283–1288.

106 S.D. Olson, A. Kambal, K. Pollock, *et al.* Examination of mesenchymal stem cell-mediated RNAi transfer to Huntington's disease affected neuronal cells for reduction of huntingtin. *Mol. Cell. Neurosci.* 2012;**49**:271–281.

107 A.A. Avilion, S.K. Nicolis, L.H. Pevny, *et al.* Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev.* 2003;**17**:126–140.

108 J. Nichols, B. Zevnik, K. Anastassiadis, *et al.* Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. *Cell* 1998;**95**:379–391.

109 A.C. Bachoud-Lévi. Neural grafts in Huntington's disease: viability after 10 years. *Lancet Neurol.* 2009;**8**:979–981.

110 A.C. Bachoud-Lévi, P. Rémy, J.P. Nguyen, *et al.* Motor and cognitive improvements in patients with Huntington's disease after neural transplantation. *Lancet* 2000;**356**:1975–1979.

111 A.C. Bachoud-Lévi, V. Gaura, P. Brugières, *et al.* Effect of fetal neural transplants in patients with Huntington's disease 6 years after surgery: a long-term follow-up study. *Lancet Neurol.* 2006;**5**:303–309.

112 F. Cicchetti, S. Saporta, R.A. Hauser, *et al.* Neural transplants in patients with Huntington's disease undergo disease-like neuronal degeneration. *Proc. Natl. Acad. Sci. U. S. A.* 2009;**106**:12483–12488.

113 Keene C.D., R.C. Chang, J.B. Leverenz, *et al.* A patient with Huntington's disease and long-surviving fetal neural transplants that developed mass lesions. *Acta Neuropathol. (Berl.)* 2009;**117**: 329–338.

114 I. Reuter, Y.F. Tai, N. Pavese, *et al.* Long-term clinical and positron emission tomography outcome of fetal striatal transplantation in Huntington's disease. *J. Neurol. Neurosurg. Psychiatry* 2008;**79**: 948–951.

115 J. Altman and G.D. Das. Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats. *J. Comp. Neurol.* 1965;**124**:319–335.

116 C. Lois and A. Alvarez-Buylla. Long-distance neuronal migration in the adult mammalian brain. *Science* 1994;**264**:1145–1148.
117 B.A. Reynolds and S. Weiss. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* 1992;**255**:1707–1710.
118 L. Conti, E. Reitano, and E. Cattaneo. Neural stem cell systems: diversities and properties after transplantation in animal models of diseases. *Brain Pathol*. 2006;**16**:143–154.
119 J.K. Ryu, J. Kim, S.J. Cho, *et al*. Proactive transplantation of human neural stem cells prevents degeneration of striatal neurons in a rat model of Huntington disease. *Neurobiol. Dis.* 2004;**16**:68–77.
120 S.T. Lee, J.-E. Park, K. Lee, *et al*. Noninvasive method of immortalized neural stem-like cell transplantation in an experimental model of Huntington's disease. *J. Neurosci. Methods* 2006;**152**:250–254.
121 C.R. Yang and R.K. Yu. Intracerebral transplantation of neural stem cells combined with trehalose ingestion alleviates pathology in a mouse model of Huntington's disease. *J. Neurosci. Res.* 2009;**87**: 26–33.
122 E.M. Vazey, K. Chen, S.M. Hughes, and B. Connor. Transplanted adult neural progenitor cells survive, differentiate and reduce motor function impairment in a rodent model of Huntington's disease. *Exp. Neurol.* 2006;**199**:384–396.
123 C. Bernreuther, M. Dihné, V. Johann, *et al*. Neural cell adhesion molecule L1-transfected embryonic stem cells promote functional recovery after excitotoxic lesion of the mouse striatum. *J. Neurosci.* 2006;**26**:11532–11539.
124 M. Bosch, J.R. Pineda, C. Suñol, *et al*. Induction of GABAergic phenotype in a neural stem cell line for transplantation in an excitotoxic model of Huntington's disease. *Exp. Neurol.* 2004; **190**:42–58.
125 J.R. Pineda, N. Rubio, P. Akerud, *et al*. Neuroprotection by GDNF-secreting stem cells in a Huntington's disease model: optical neuroimage tracking of brain-grafted cells. *Gene Ther*. 2007;**14**:118–128.
126 J. Song, S.-T. Lee, W. Kang, *et al*. Human embryonic stem cell-derived neural precursor transplants attenuate apomorphine-induced rotational behavior in rats with unilateral quinolinic acid lesions. *Neurosci. Lett.* 2007;**423**:58–61.
127 V. Johann, J. Schiefer, C. Sass, *et al*. Time of transplantation and cell preparation determine neural stem cell survival in a mouse model of Huntington's disease. *Exp. Brain Res.* 2007;**177**:458–470.
128 J. Rossignol, K. Fink, K. Davis, *et al*. Transplants of adult mesenchymal and neural stem cells provide neuroprotection and behavioral sparing in a transgenic rat model of Huntington's disease. *Stem Cells* 2014;**32**:500–509.
129 M. Kawakami, T. Yoshimoto, N. Nakagata, *et al*. Effects of cyclosporin A administration on gene expression in rat brain. *Brain Inj*. 2011;**25**:614–623.
130 K. Takahashi and S. Yamanaka. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–676.
131 HD iPSC Consortium. Induced pluripotent stem cells from patients with Huntington's disease show CAG-repeat-expansion-associated phenotypes. *Cell Stem Cell* 2012;**11**:264–278.
132 K.D. Fink, A.T. Crane, X. Lévêque, *et al*. Intrastriatal transplantation of adenovirus-generated induced pluripotent stem cells for treating neuropathological and functional deficits in a rodent model of Huntington's disease. *Stem Cells Transl. Med.* 2014;**3**:620–631.
133 J. Rossignol, A.T. Crane, K.D. Fink, and G.L. Dunbar. Will undifferentiated induced pluripotent stem cells ever have clinical utility? *J. Stem Cell Res. Ther.* 2014;**4**:100189.
134 H. Kawai, T. Yamashita, Y. Ohta, *et al*. Tridermal tumorigenesis of induced pluripotent stem cells transplanted in ischemic brain. *J. Cereb. Blood Flow Metab.* 2010;**30**:1487–1493.
135 T. Yamashita, H. Kawai, F. Tian, *et al*. Tumorigenic development of induced pluripotent stem cells in ischemic mouse brain. *Cell Transplant*. 2011;**20**:883–891.
136 Y. Zhang, D. Wang, M. Chen, *et al*. Intramyocardial transplantation of undifferentiated rat induced pluripotent stem cells causes tumorigenesis in the heart. *PloS ONE* 2011;**6**:e19012.
137 K.D. Fink, J. Rossignol, M. Lu, *et al*. Survival and differentiation of adenovirus-generated induced pluripotent stem cells transplanted into the rat striatum. *Cell Transplant*. 2014;**23**:1407–1423.
138 T.J. Nelson, A. Martinez-Fernandez, S. Yamada, *et al*. Repair of acute myocardial infarction by human stemness factors induced pluripotent stem cells. *Circulation* 2009;**120**:408–416.
139 K.Y. Yang, H.-C. Shih, C.-K. How, *et al*. IV delivery of induced pluripotent stem cells attenuates endotoxin-induced acute lung injury in mice. *Chest* 2011;**140**:1243–1253.
140 I. Jeon, N. Lee, J.-Y. Li, *et al*. Neuronal properties, *in vivo* effects, and pathology of a Huntington's disease patient-derived induced pluripotent stem cells. *Stem Cells* 2012;**30**:2054–2062.
141 I. Jeon, C. Choi, N. Lee, *et al*. *In vivo* roles of a patient-derived induced pluripotent stem cell line (HD72-iPSC) in the YAC128 model of Huntington's disease. *Int. J. Stem Cells* 2014;**7**:43–47.

SECTION IV

# The role of bioengineering in the therapeutic applications of mesenchymal stromal cells

CHAPTER 41

# Endometrial mesenchymal stromal cell and tissue engineering for pelvic organ prolapse repair

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## 41.1 Introduction

Pelvic organ prolapse (POP) is a very common condition affecting millions of women worldwide. Recent notifications by the US Food and Drug Administration (FDA) warning of the high rate of complications with transvaginal placement of polypropylene (PP) mesh have led to withdrawal of many vaginal meshes from the market. This now severely limits the available treatment options for large numbers of women with POP. There is an urgent need for an effective therapy for this major hidden disease burden of women. This chapter will review recent studies in an emerging field in regenerative medicine that suggest a tissue engineering (TE) approach for the treatment of POP. It will detail new mesh designs that are more likely to succeed than the present PP meshes. It will describe the potential of cell-based therapy for POP, highlighting a newly identified source of mesenchymal stem/stromal cells (MSCs) from human endometrium. Characterization of endometrial MSCs (eMSCs) and the use of specific markers for their enrichment will be discussed. Approaches undertaken to produce eMSCs under good manufacturing practice conditions will be emphasized. Finally, the need for a large animal model of vaginal repair that can assess new TE constructs for a longer duration will be highlighted as a necessary step before the first clinical trials of a cell-based therapy can be commenced in women with POP. Figure 41.1 shows the anatomy of the human uterus, fallopian tubes, ovaries and vagina.

The uterus is made up of an external layer of smooth muscle called the myometrium and an internal layer called the endometrium. The endometrium has three layers: the stratum compactum, the stratum spongiosum (which make up the stratum functionalis), and the stratum basalis. The stratum compactum and stratum spongialis develop into the stratum functionalis during the first half of the menstrual cycle (proliferative phase).

## 41.2 Pelvic floor disorders

Pelvic floor disorders (PFDs) encompass stress urinary incontinence, fecal incontinence, and POP. The overall

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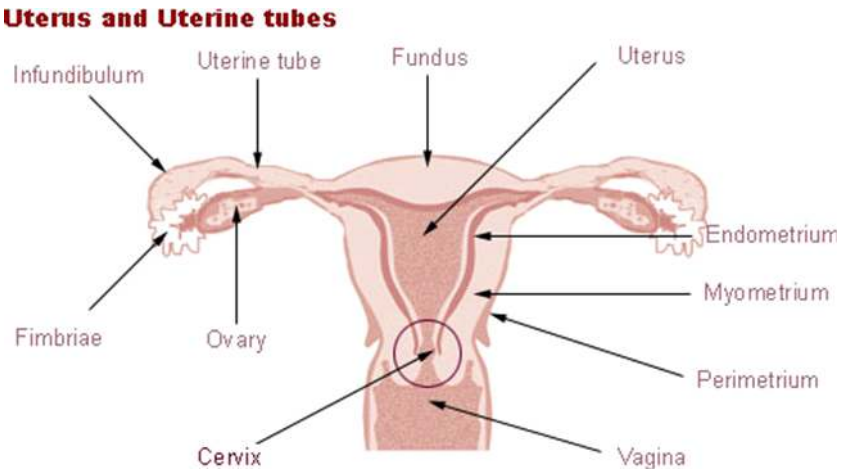



**Figure 41.1** Anatomy of the human uterus, fallopian tubes, and vagina. This work is in the public domain in the USA because it is a work prepared by an officer or employee of the United States Government as part of that person's official duties under the terms of Title 17, Chapter 1, Section 105 of the US Code. http://training.seer.cancer.gov/ss_module09_cervix_corpus/cervix_unit01_sec01_intro.html.

prevalence of PFD in women is 35%, which increases to 52% for women above 70 years of age [1–3]. Women as young as 18 years of age are affected by this condition, and the prevalence increases significantly with age [4,5]. Major risk factors include pregnancy, vaginal delivery, and obesity. Although many pregnant women seek elective cesarean section to prevent PFDs, compelling evidence suggests that the incidence of PFD in women undergoing this elective procedure does not decrease compared with nulliparous women or those undergoing vaginal birth delivery [1,6].

## 41.3 Pelvic organ prolapse

POP is a major hidden burden affecting millions of women worldwide. POP is the downward descent or herniation of pelvic organs, including the bladder, bowel, and/or uterus, into the vagina due to a weakened or damaged pelvic floor support structures [7]. POP accounts for over two-thirds of PFDs [7]. The support structures of female pelvic organs comprise the endopelvic fascia of the vaginal wall, the suspensory ligaments, and the levator ani muscles (pelvic floor muscles) [3,7]. Pregnancy and vaginal delivery can injure these support structures, through damage or denervation to muscles [8], overstretching or tearing of the ligaments, and weakening of the endopelvic fascia. In addition to the immense stretching of the pelvic floor during vaginal delivery, 10–20% of women giving birth to their first child have prolonged labor, instrument delivery, a large infant weight, or an episiotomy, all of which contribute to pelvic floor injury leading to POP [9]. Subsequent pregnancies and child birth and aging further weaken these tissues. Symptoms of POP include urinary incontinence, bowel incontinence, and/or sexual dysfunction [9].

One in four women suffer from POP, for which surgical reconstruction is frequently required. Women have an 11–20% probability of requiring an operation for POP repair in their lifetime [4,10–12]. POP is the leading indication for hysterectomy in postmenopausal women [13], and in Australia approximately 22 000 POP repair operations are conducted every year. In the USA the figure is 300 000 per year [7]. Although the lifetime risk for POP is higher than breast or lung cancer [14], most women suffer in silence because of the embarrassing symptoms [15]. A survey showed that only one in three US women had reluctantly discussed bladder issues with their doctor because of a perceived stigma and associated embarrassment. With increasing rates of obesity, delayed first childbirth, and rising life expectancy, the disease burden related to POP in terms of decreased quality of life, social isolation, lost productivity, and the effects on families and health-care systems will be substantial [3]. A comparative study in the USA estimated that 17 million women were affected by urinary incontinence, and the cost was estimated to be $19.5 billion [16]. Long-term, safe, and more effective therapies and earlier interventions are needed for affected women in order to improve their quality of life.

### 41.3.1 Surgical treatment for pelvic organ prolapse

Depending on which organs and support structures are affected and the severity of POP, the ultimate treatment is reconstructive surgical repair of the vaginal wall transvaginally (colporrhaphy). Native tissue surgical treatment has a high failure rate, with 30% of cases requiring further surgery for recurrent POP and with the time frame between surgeries decreasing with increasing age [4]. This high failure rate of native tissue repair prompted clinicians to adapt commercial synthetic abdominal hernia meshes for POP repair. These meshes aimed to provide additional support of the damaged pelvic tissues and to improve long-term outcomes. Synthetic PP meshes were introduced in 1997 and gained FDA approval in 2004. Since then, surgical methods for repair of POP have increased in number and complexity. In the USA, mesh had

been used in a third of POP surgeries and inserted transvaginally in ~75% of cases [17]. These mesh-augmented repair operations delivered seemingly higher "cure" rates, particularly for anterior vaginal wall damage, compared with colporrhaphy using native tissue surgery [18–20]. More recently, however, serious complications, such as mesh exposure into the vagina, bladder, or bowel, mesh shrinkage, vaginal shortening, pain, dyspareunia, and infections, have been reported in as many as 10–15% of cases [21–23]. This alarming complication rate led to two notifications by the FDA in 2008 and 2011 warning of the complications with transvaginal placement of surgical mesh [17,24]. These notifications have led to the withdrawal of some vaginal meshes from the market by several leading device companies. There is now a real risk that successful treatment options for women will be reduced and ineffective for a large number of women with POP.

### 41.3.2 New meshes for treatment of pelvic organ prolapse

Meshes used for clinical purposes may either be biological or synthetic. Biological scaffolds are typically natural tissues such as autologous rectal fascia and fascia lata (the deep fascia of the thigh that encloses the thigh muscles), allogeneic, such as cadaveric tissue, or decellularized xenografts, such as porcine small intestinal submucosa (SIS). Synthetic meshes, predominantly fabricated from PP, are the most commonly used for POP repair. Biological materials can be used as an untreated, decellularized matrix or can be further stabilized by chemical cross-linking. In either case, they are largely biodegradable and have limited efficacy due to their variable degradation rate, lack of longevity, variable tissue integration and host response, and concerns over transmission of diseases. Synthetic meshes are often preferred over biological meshes for applications requiring long-term support of tissues because they have more consistent properties and there is a more predictable host response [25]. While currently used PP meshes provide good mechanical tensile support for the damaged vaginal wall, they are generally stiffer than the natural tissue, resulting in compromised distensibility, poor compliance, and less integration into vaginal fascial tissues [26]. In addition, synthetic materials, while normally inert and nonimmunogenic, can induce a relatively vigorous foreign-body reaction leading to fibrotic tissue deposition [27].

The ideal mesh for POP has not yet been designed [28]. Adverse effects associated with current meshes can lead to erosion where the implanted material protrudes into the vaginal, bladder, or bowel lumen. POP meshes should be biocompatible and provide sufficient mechanical strength to support damaged tissue, but should also be elastic and flexible enough to allow compliance, movement, and integration with the natural tissue [25,26].

The physical properties of mesh depend upon polymer composition of the material, filament type, weaving or knit pattern, pore size, weight, and coating [26,27]. The current range of meshes will vary in one or more of these properties. Evolution of mesh design has largely focused on reduced weight with accompanying large pore diameter, which is conducive to cell infiltration, new fibro-collagenous deposition, and tissue remodeling [26,29].

Our recent work has used these current design strategies to further improve the biomechanical and biological performance of new fabricated polymer mesh types. The *in vitro* structural and mechanical properties of three commercially available PP meshes were compared with these new fabricated meshes from alternative polymers [26]. One of these meshes fabricated from polyamide (PA) monofilament yarn was optimized with a pore size of 1000 μm and dip-coated with 12% porcine gelatin (PA/G) to provide a platform for delivering cells to the surgical site (Figure 41.2D). The new PA/G meshes were less stiff on uniaxial biomechanical testing, more flexible and elastic on multiaxial testing, and with lower bending rigidity than commercial PP meshes. These favorable properties allow the mesh to conform to the highly mobile human vaginal tissue, with a less predictable likelihood to erode into neighboring organs. *In vivo* evaluation of the fibronectin-coated PA/G mesh designs in a rat abdominal hernia model showed significantly improved tissue integration by promoting angiogenesis, exerting a moderate foreign body reaction and generating good collagen deposition for up to 90 days compared with commercial PP mesh [27]. This new mesh with superior biomechanical and biocompatibility properties may be a more suitable starting point for developing a TE construct for POP therapy.

## 41.4 Tissue engineering

TE is an emerging field in regenerative medicine that uses a combination of cells and biomaterials of synthetic or natural origin (Figure 41.2D and E) The goal is to replace or regenerate damaged or diseased human tissues and restore or improve the long-term outcomes of surgical therapy [26]. In the urogynecological field, preclinical studies using animal models and clinical trials are scarce and have predominantly focused on the use of cell-based therapies for stress urinary incontinence. There are few studies that have explored the potential use of cell-based or TE approaches as potential therapies for POP. We are proposing to use a novel cell source with newly designed meshes to generate a TE approach for treating women with POP (Figure 41.2). The following summarizes the *in vitro* studies and studies in preclinical animal models (Figure 41.2F).

### 41.4.1 Candidate cells for tissue engineering applications for pelvic organ disorders

#### 41.4.1.1 Skeletal muscle-derived cells and muscle fibers

Murine skeletal muscle-derived stem cells (MDSCs) can differentiate into smooth muscle cells (SMCs) *in vitro* and express the pluripotency genes *OCT4* and *NANOG* [30]. MDSCs were grown on porcine SIS (Cook, Biotech®) and on a polymeric Vicryl mesh, and were found to stimulate vaginal tissue repair with reduced fibrosis in a rat model. It was postulated that MDSCs had

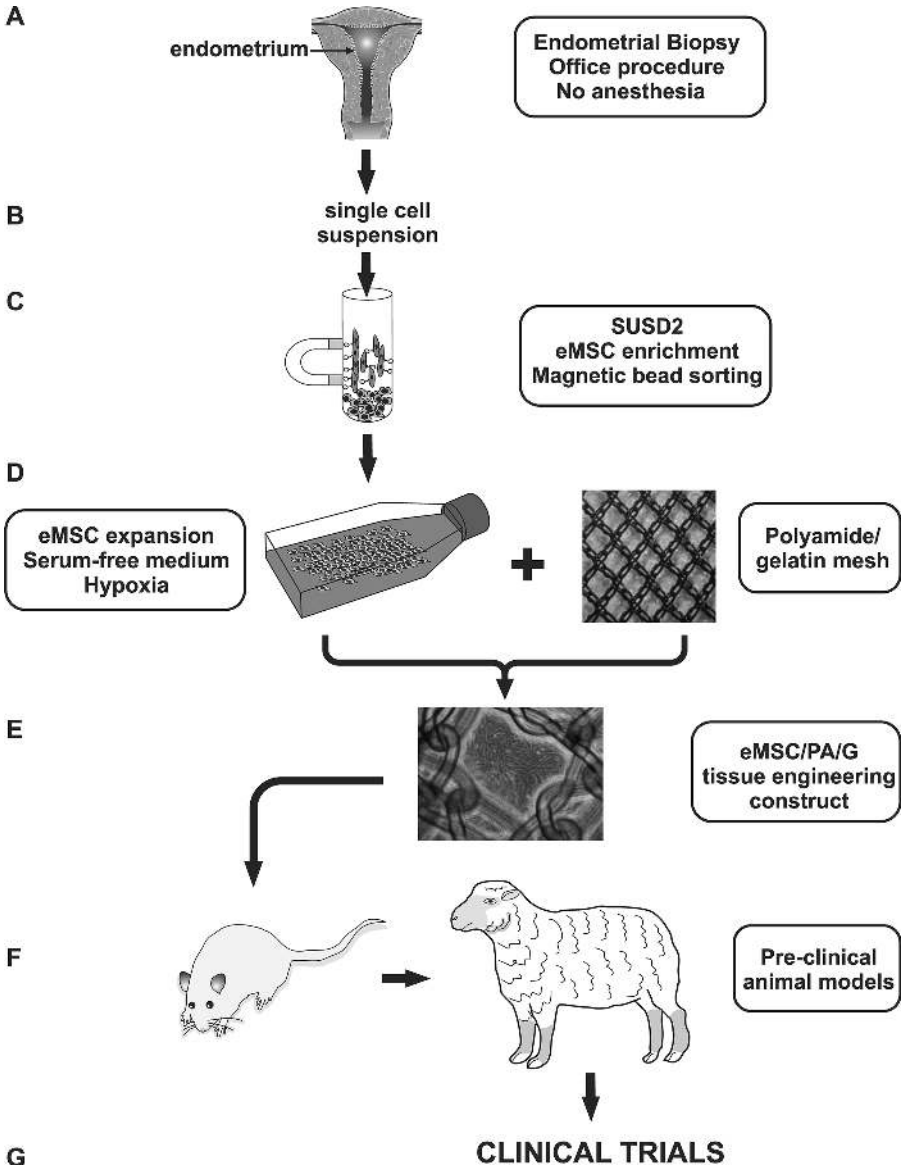



**Figure 41.2** Schematic showing a TE approach for the treatment of POP with eMSCs. Endometrium is the mucosal lining of the uterus. It can be collected (A) by biopsy in an office-based procedure without the need for anesthesia. (B) Endometrial tissue is mechanically and enzymatically dissociated into a single-cell suspension and (C) eMSCs immunoselected using SUSD2 (W5C5) antibody by magnetic bead sorting. (D) eMSCs are culture expanded in serum-free medium in physiological (5% $O_2$) hypoxia and (E) seeded on fibronectin-coated PA/G composite mesh, which is cultured for a further 48 h before transplanting into (F) small animal models (rats) and a preclinical large animal model (sheep) for proof of concept and then (G) human clinical trials.

transdifferentiated into SMCs and stratified keratin $5^+$ squamous epithelium when implanted into the injured vaginal tissue as a TE construct. α-smooth muscle actin (α-SMA) was used to evaluate the resulting tissue generated, but it could not discriminate between SMCs and myofibroblast differentiation. This is important, because the latter mediates the fibrotic process. In addition, it is unclear whether the differentiated cells had arisen from a stem cell precursor or a mixed population of heterogeneous cells. Further studies on the functional properties of the implants and their effects *in vivo* are required.

Fresh skeletal muscle fibers or cultured autologous skeletal-muscle-derived cells have also been seeded on synthetic biodegradable methoxy(polyethylene glycol)–poly(lactic–co-glycolic acid) meshes and implanted subcutaneously into the rat abdomen [31]. The TE constructs were well tolerated, and in contrast to the previous study the cultured skeletal muscle cells had disappeared by 8 weeks. However, there was generation of fragmented striated muscle tissue in the skeletal muscle fiber tissue construct [31], indicating superiority of fresh skeletal muscle fibers to culture-expanded skeletal muscle cells for surgical reconstruction in an animal abdominal wall model of POP repair surgery [31].

#### 41.4.1.2 Fibroblasts

Human vaginal fibroblasts have also been suggested as an alternative cell source for a TE approach to treat POP. Vaginal

fibroblasts were expanded, added to a collagen solution, labeled with the fluorescent dye DiL and applied to biodegradable poly (D,L-lactic–co-glycolic acid) meshes [32]. The TE construct was implanted subcutaneously into mice, where a well-organized multilayered neofascia of collagen was generated. The DiL-labeled cells survived and were identified within the construct after 12 weeks, indicating the contribution from the human vaginal fibroblasts.

The feasibility of using autologous vaginal fibroblasts for the treatment of POP is attractive; however, POP vaginal fibroblasts are less than ideal. Vaginal fibroblasts from the prolapsed site in women with POP showed delayed collagen contractility and lower matrix metalloproteinase-2 production compared with fibroblasts from normal premenopausal women [33]. The immunoreactive collagen and elastin scores of the vaginal connective tissue from women with POP were higher than with normal vaginal tissue but contained fewer fibroblasts [34]. This increased collagen staining does not accurately reflect total collagen content, nor does it take into account alterations in the ratio of type III/type I collagen. A higher ratio, as found in women with POP, is characterized by mechanically inferior tissue, suggesting that pathological changes in vaginal wall connective tissue composition changes the quality and the functional ability of the vaginal fibroblasts, and this should be considered when opting to use them [33].

Buccal mucosal fibroblasts are a readily available cell source for TE. They express the typical MSC/fibroblast surface markers, but do not differentiate into adiopogenic and chondrocyte lineages and have limited osteogenic differentiation ability. Despite this, buccal mucosal fibroblasts produce collagen I *in vitro*, an important component of extracellular matrix [35]. Cultured buccal fibroblasts attach well in heat-annealed poly(L-lactic acid) (PLA) scaffold, produce collagen and elastin under intermediate stress forces *in vitro*, and have been suggested as a cell type for use in the treatment of POP [36]. However, *in vivo* work in an appropriate animal model is required to assess the utility and long term effects of buccal fibroblasts for tissue repair.

#### 41.4.1.3 Mesenchymal stem/stromal cells

MSCs were first discovered in the bone marrow as an adherent, clonogenic, nonhematopoietic cell population that undergoes extensive proliferation in culture and differentiates into multiple mesodermal lineages, including fat, bone, cartilage, stroma, tendon, and muscle [37]. MSCs have since been identified in multiple organs, including adipose tissue, dental pulp, umbilical cord tissue, placenta, and endometrium [38,39]. MSCs are an attractive source of cells for regenerative medicine, as evidenced by the ~400 clinical trials now listed on clinical trial websites (such as ClinicalTrials.gov) and the 2000 or more patients who have received varying forms of MSC as experimental therapy without any serious adverse events [40,41]. A systematic review and meta-analysis of clinical trials involving MSCs given intravenously or intra-arterially showed systemically delivered MSCs to be safe [41]. Thus far there have been no clinical trials of MSC or cell-based therapy for POP. MSCs home to sites of tissue damage and act in a paracrine manner, secreting bioactive signaling molecules that promote angiogenesis, limit fibrosis, enhance anti-inflammatory and immune suppressive function, inhibit apoptosis, and induce tissue-specific progenitor cells to proliferate – processes that collectively repair damaged tissue [40,42]. In preclinical animal models, MSCs have also been delivered directly to damaged tissue in scaffolds as TE constructs for repairing bone, cartilage, fat, tendon, muscle, and connective tissues. Scaffolds provide structural support and sites for cell adhesion, allowing delivery and retention of large numbers of cells at the site of tissue injury [43]. In this mode the MSCs integrate and proliferate in the neotissue and/or have paracrine effects, modulating tissue repair without long-term engraftment [44]. MSCs seeded on to a scaffold deliver larger doses of secreted bioactive molecules locally to an injury site compared with systemic delivery of MSCs [42]. Preclinical studies are required to determine the efficacy of MSCs, their mechanism of action, and whether MSC-seeded materials are superior in regenerating tissues than materials alone [40].

In order to provide consistency among investigators in the definition of MSCs, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed minimal criteria in 2007 to minimize experimental variability in MSC research [45]. MSCs should be more than 95% positive for the surface markers CD90, CD105, and CD73 and negative for the hematopoietic lineage markers CD45, CD14, and CD11 as well as for the co-stimulatory molecules CD80 and CD40. However, MSCs defined by such methods still include heterogeneous populations with different levels of "stemness." Thus, the markers defined above are not suitable for the prospective isolation of MSCs from tissues. Furthermore, MSCs undergo spontaneous replicative differentiation and senescence during culture expansion, losing their stemness and regenerative ability despite expression of the listed phenotypic markers [46,47]. These markers fail to discriminate between undifferentiated MSCs and differentiated mature fibroblasts (see Chapter 30). Culture conditions, including seeding density, confluency, and growth factors, also have the potential to influence MSC phenotype marker expression.

Clinical translation of MSCs for cell-based therapies includes autologous and allogeneic sources. For autologous use, the easy accessibility of a cell source is vital. Common sources, such as bone marrow and adipose tissue biopsy, involve painful procedures for procurement and require some form of anesthesia. These sources often produce low yields. A new source of MSCs from human endometrium (i.e., eMSCs) has been proposed as an alternative autologous (or allogeneic) source because of their easy accessibility (Figure 41.2E). eMSCs can be obtained via endometrial biopsy in an office-based procedure, incurring minimal pain, scarring, or morbidity, and generally do not require an anesthetic [48–50].

## 41.5 Endometrium is highly regenerative and contains stem/stromal cells

The endometrium or mucosal lining of the uterus is a dynamic tissue. Following menstruation as much as 10 mm of endometrium is regenerated in the first half of the subsequent menstrual cycle. The endometrium then differentiates in preparation to receive an embryo in the second half of the cycle, and sheds if there is no pregnancy [51,52]. This repeats for 400–500 cycles of growth, differentiation, and shedding during a woman's reproductive life. Endometrial regeneration also occurs in the postpartum period and in postmenopausal women taking estrogen therapy [53,54]. This level of cellular turnover and scarless mucosal regeneration in the endometrium is similar to that observed in other highly regenerative tissues, such as the skin, gastrointestinal tract, and bone marrow [52], where resident stem/progenitor cells have known roles in maintaining tissue homeostasis [50].

### 41.5.1 Human endometrial mesenchymal stem/stromal cells

Key properties of any stem cell include the ability to form colonies from single cells, to self-renew into daughter stem cells, and to differentiate into one or more lineages and reconstitute tissue *in vivo*. Colony-forming unit (CFU) cells were first identified in stromal cell suspensions from human endometrium in 2004 [55]. These CFUs–fibroblast (CFU-Fs) comprised 1.25% of stromal cells. There was a range of clone sizes, but only the rarer large CFUs demonstrated classic MSC properties: self-renewal *in vitro* assessed in serial cloning assays, mesodermal lineage differentiation to adipocytes, chondrocytes, osteoblasts, and myocytes, and a characteristic surface phenotype [56]. There were variations in the clonogenic activity at different stages of the menstrual cycle and in noncycling endometrium, but these differences were not statistically significant [57]. Nonetheless, 0.02% of freshly isolated endometrial stromal cells generated large colonies. Cells initiating a large colony could be serially cloned four or five times at low seeding densities (10 cells/cm$^2$) before CFU activity was exhausted, indicating extensive self-renewal capacity *in vitro* [56]. Single cells initiating the large colonies also demonstrated high proliferative ability, undergoing 30 population doublings and generating several billion cells. The culture-expanded cells from the large colonies expressed the characteristic but not unique MSC markers CD29, CD44, CD73, CD105, and CD146 and were negative for CD45, CD34, CD31, and stromal precursor antigen-1 (Stro-1) [56]. Freshly isolated endometrial stromal cells also generated colonies in serum-free medium supplemented with fibroblast-derived growth factor (FGF)-2, epidermal growth factor, platelet-derived growth factor (PDGF)-BB, or transforming growth factor (TGF)-α, although the cloning efficiency was lower in the absence of serum [55]. These findings indicate that multiple factors are likely to act as niche factors *in vivo*.

#### 41.5.1.1 Prospective isolation and functional properties of human endometrial mesenchymal stem/stromal cells

Efforts are in progress to find markers to identify MSCs in many tissues and in different species. Identification of markers for the prospective isolation of MSCs is important in order to demonstrate their location in tissues, characterize their properties and genomic signature, and to harness them for their therapeutic properties. To identify markers of human endometrial stromal CFUs, stromal cells were flow-sorted using four commonly used MSC markers (CD90, CD146, CD133, and Stro-1) and examined for CFU activity [58]. CD90$^{high}$ and CD146$^{+}$ individually enriched for stromal CFUs and immunostaining identified their perivascular location in human endometrium. Stro-1, a bone marrow MSC marker, did not enrich for endometrial stromal CFUs despite its perivascular location. Importantly, endometrial stromal cells co-expressing two markers, CD140b and CD146, contained almost all stromal CFUs and accounted for 1.5% of the total stromal cells [38]. Single CD140b$^{+}$ CD146$^{+}$ cells underwent multilineage differentiation into the typical MSC mesodermal lineages of adipocytes, chondrocytes, osteocytes, and myocytes [38]. They had the characteristic MSC surface marker phenotype and were negative for the hematopoietic markers CD34, CD31, and CD45. Together, these data showed that almost all eMSCs are found in the CD140b$^{+}$ CD146$^{+}$ subpopulation. Examining the co-expression of these two markers by immunofluorescence revealed the perivascular location of eMSCs in the layer that is shed at menstruation and in the remaining basal layer of human endometrium. Their close association with endothelial cells pointed to the likely pericyte identity of eMSCs. It was also noted that the CFU ability of the cells following flow cytometry was threefold less compared with unsorted cells, indicating that flow cytometry sorting adversely affects cell viability [58]. Therefore, it was important to find a single marker of eMSCs to enable a simpler method of enrichment using magnetic bead sorting (Figure 41.2C) [59]. Screening endometrial stromal cell suspensions with several novel antibodies with perivascular specificity [60] by flow cytometry and immunohistochemistry identified a single marker detected by the W5C5 antibody [59].

The W5C5 antibody targets the extracellular domain of Sushi domain containing 2 (SUSD2), a type I transmembrane adhesion molecule. SUSD2 has a large extracellular region containing a somatomedin B, adhesion-associated domain in MUC4 and other proteins (AMOP), a von Willebrand factor (vWF), and a Sushi domain. Although the biological function of SUSD2 is not fully known, somatomedin B, AMOP, and von Willebrand factor domains are components of proteins that participate in cell adhesion, homodimerization, migration, homing, and signal transduction [60]. Large-scale mapping of human protein–protein interactions by mass spectrometry indicates that SUSD2 may be involved in cell-to-cell binding [61]. Clonogenic bone marrow MSCs reside in the CD271$^{bright}$ but not in the CD271$^{dim}$ population. Furthermore, the W5C5 antibody

exclusively identified the CD271$^{bright}$ bone marrow MSCs but not the dim population [62]. Apart from functional and phenotypic differences, the CD271$^{bright}$/W5C5$^+$ bone marrow MSCs have bright nuclear staining compared with the dark immature lymphoblastic appearance of the CD271$^{dim}$ cells. This supports W5C5 as a promising marker candidate for isolating MSCs.

As with bone marrow MSCs, the majority of endometrial CFU-Fs are found in the W5C5$^+$ fraction. W5C5$^+$ magnetic-bead-sorted endometrial stromal cells generated more colonies than flow-cytometry-sorted W5C5$^+$ cells and flow-cytometry-sorted CD140b$^+$ CD146$^+$ cells, indicating the utility of magnetic bead sorting with a single marker. Exploring the relationship between W5C5 and existing eMSCs markers showed that 85% of fresh W5C5$^+$ cells expressed CD140b and 60% expressed Stro-1, while all the W5C5$^+$ CD146$^+$ cells were positive for CD140b. There was no difference in the colony-forming ability between the W5C5$^{hi}$ and W5C5$^+$ CD146$^+$ cells [59]. W5C5$^+$ stromal cells demonstrated typical MSC properties: mesodermal lineage differentiation into Oil Red O-stained adipocytes that expressed peroxisome proliferator-activated receptor gamma 2, osteogenic cells which stained for alkaline phosphatase and expressed core binding factor-α1, also known as RUNX, and chondrocytes surrounded by Alcian blue-positive cartilage matrix expressing collagen X. In addition, W5C5$^+$ endometrial stromal cells differentiated into early stage SMCs staining for α-SMA as well as later differentiation stages by staining for smooth muscle myosin heavy chain and to CD31$^+$ endothelial cells [59]. The surface phenotype of the W5C5$^+$ beaded population showed that they predominantly expressed CD146 and CD90, endometrial perivascular and stromal markers respectively [59]. The identification of CD140b$^+$ CD146$^+$ and W5C5$^+$ as potential markers of eMSCs and their presence in the endometrial layer that is shed during menses indicates that eMSCs can be harvested from endometrial biopsy samples as well as from menstrual blood [50]. The ultimate proof of the regenerative capability of stem/progenitor cells is their ability to reconstitute the parent tissue from which they were derived. Human vimentin-positive endometrial stromal-like tissue was generated when W5C5$^+$ eMSCs were transplanted under the kidney capsule of severely immunodeficient NOD SCID GAMMA mice [59]. In addition to the stromal-like tissue, migratory cells were also observed in the mouse kidney parenchyma, which were morphologically similar to CD31$^+$ endothelial cells demonstrated in xenografts from endometrial side population cells [63]. Therefore, both *in vitro* and *in vivo* studies have demonstrated that W5C5$^+$ eMSCs differentiate into endometrial fibroblasts and vascular cells [59,64].

Other surface markers have also been identified for isolating eMSC-like cells. Mesenchymal stem cell antigen-1 (MSCA-1) is a novel molecule on the surface of MSCs that is suitable for their prospective isolation [65]. The molecular identity of MSCA-1 is tissue nonspecific alkaline phosphatase (TNAP) [65]. TNAP/MSCA-1 co-localized with a subpopulation of CD146$^+$ endometrial cells at a similar percentage as for CD146$^+$ CD140b$^+$ eMSCs [58], but not in differentiated stromal fibroblasts from bone marrow or endometrium. TNAP/MSCA-1 is not a suitable single marker for isolating eMSCs as immuno-localization showed not only endometrial perivascular cell staining but also the luminal surface of the glandular epithelium [65]. However, co-expression of TNAP/MSCA-1 with CD146 can be used to prospectively sort and isolate eMSCs.

All specific markers identified for eMSCs (CD140b$^+$, CD146$^+$, SUSD2, and TNAP/CD146) show that eMSCs are present in both stratum functionalis and stratum basalis layers of the endometrium and are located perivascularly. Thus, it was anticipated that menstrual blood would also contain a subpopulation of eMSCs. Indeed, adherent menstrual blood cells cultured for five passages have been immunoselected with CD117 (c-kit) beads but yielded only 9% [66]. Further culturing for three more passages increased this yield to 77% of CD117$^+$ cells. However, the existence of fresh c-kit-expressing stromal cells in menstrual blood still remains to be demonstrated. Immunostaining of endometrial tissue over the lifespan from the female fetus to the atrophied tissue of postmenopausal women demonstrated that the CD117$^+$ stromal cells were present as scattered single cells in the endometrial stroma and myometrial stroma, but mostly in the endometrial glands in adult life [67]. As with TNAP, CD117 is also expressed on endometrial glandular epithelial cells. Thus, a simple isolation procedure of eMSCs without epithelial contamination is not possible with TNAP or CD117.

#### 41.5.1.2 Endometrial mesenchymal stem/stromal cell phenotype and gene expression profile

Gene expression profiling on freshly isolated endometrial stromal cells sorted using flow cytometry on the basis of CD146 and CD140b expression has provided insight into the global state of eMSCs' gene expression and their inferred functions [64]. The three cell types compared were CD146$^+$ CD140b$^+$ (eMSCs), CD146$^-$ CD140b$^+$ (fibroblasts), and CD146$^+$ CD140b$^-$ (endothelial cells) [64]. Hierarchical clustering and principle component analysis showed greater similarity of gene profiles between eMSCs and stromal fibroblasts, which were distinct from endothelial cells. Despite the relatively similar profiles of eMSCs and endometrial fibroblasts, 762 genes were differentially expressed between these cell types, approximately half the number of differences observed between eMSCs and endometrial endothelial cells. This study indicated a relatively close relationship between eMSCs and endometrial stromal fibroblasts, and that endometrial fibroblasts are a lineage cell of eMSCs. Importantly, the CD146$^+$ CD140b$^+$ population expressed significantly greater levels of the single marker SUSD2 now used to enrich for eMSCs [68].

The pericyte phenotype of eMSCs was confirmed by the expression of pericyte markers such as the regulator of G-protein signaling (*RGS5*), α-smooth muscle actin-2 (*ACTA2*), angiopoietin 2 (*ANGPT2*), insulin-like growth factors (*IGFs*), nerve growth factor (*NGF*), platelet-derived growth factor (*PDGF*), transforming growth factor-β (*TGF-β*), parathyroid hormone-like hormone (*PTHLH*), and syndecan. Gene families responsible for

self-renewal and differentiation, such as Notch, TGFB, Wnt, IGF, FGF, Hedgehog members, and cyclic adenosine-monophosphate-mediated and G-protein-coupled receptor-mediated signaling were also upregulated in eMSCs [64]. Furthermore, this study confirmed that endometrial CD140b$^+$ CD146$^+$ cells are clonogenic and can differentiate into mesodermal lineages.

Gene profiling of passage 2 culture-expanded SUSD2$^+$ eMSCs identified 12 perivascular genes upregulated in SUSD2$^+$ eMSCs. Perivascular markers, including melanoma cell adhesion molecule (CD146), vascular endothelial protein 1 (also known as amine oxidase, copper containing 3 (*AOC3*)), *NOTCH3*, a smooth muscle myosin (*MYTH11*), and elastin (*ELN*) were enriched in cultured SUSD2$^+$ eMSCs. These genes translated as the proteins were also identified around endometrial arterioles by immunostaining [69]. Desmin (DES), α-SMA (ACTA2), and chondroitin sulfate proteoglycan 4 (CSPG4), which are essential for regulating pericyte recruitment and sprouting during angiogenesis, were also highly expressed, indicative that the pericyte signature genes of the primary cells were retained in cultured eMSCs [68,69].

The Notch target genes *HEY2* and *HEYL*, but not *HEY1*, were found to be highly expressed in SUSD2$^+$ eMSCs compared with SUSD2$^-$ fibroblasts [69]. These *HEY2* and *HEYL* transcripts were significantly downregulated in human SUSD2$^+$ eMSCs treated with γ-secretase Notch inhibitor, DAPT. This was reflected by decreased SUSD2 mRNA expression and SUSD2$^+$ eMSC number to 53% and 64% respectively. This study showed for the first time that *SUSD2* is a downstream target gene in the Notch pathway in human eMSCs. Further knockdown and overexpression assays are required to confirm the involvement of Notch signaling in eMSC function. However, other signaling pathways may also be involved in eMSC regulation.

Gene profiling of freshly isolated CD140b$^+$ CD146$^+$ eMSCs did not detect the pluripotency genes *OCT4* (*POU5F1*), *SOX2*, and *NANOG* when compared with the National Institutes of Health-approved human embryonic stem cell line H7 using quantitative real time reverse transcriptase polymerase chain reaction [64]. In contrast, others have demonstrated the expression of OCT4 (POU5F1) but not NANOG [70] and the embryonic stem cell markers SSEA-4 and c-KIT (CD117) [66] using flow cytometry in culture-expanded endometrial regenerative cells cultured from menstrual blood. Some studies suggest that the *OCT4* gene is expressed by adult stem/progenitor cells and primarily regulates self-renewal, but others have shown that only pluripotent stem cells such as human embryonic stem cells and induced pluripotent stem cells express these pluripotency genes. There are at least three *OCT4* transcripts (*OCT4A*, *OCT4B*, and *OCT4B1*) and four protein isoforms (OCT4A, OCT4B-190, OCT4B-265, and OCT4B-164). OCT4A is the transcription factor responsible for the pluripotency properties in human embryonic stem cells, while *OCT4B* has no such role [71]. Many studies showing the expression of pluripotency genes, especially *OCT4* in adult stem cells, including endometrium, have failed to distinguish the *OCT4* isoforms or did not exclude the possibility of pseudogene artefacts [71].

Recently, much interest has focused on the soluble factors secreted by MSCs, the homing capacity of bone marrow MSCs to sites of injury, and their immune modulatory and paracrine properties. These include secretion of vascular endothelial growth factor, hepatocyte growth factor, TGF, and prostaglandin $E_2$.

A recent *in vitro* study highlighted the role of eMSCs in endometrial stromal cell decidualization (differentiation) and potentially in placentation [68]. A secretome analysis on passage 2 of SUSD2$^+$ eMSCs and SUSD2$^-$ endometrial fibroblasts following induction of decidualization (differentiation) *in vitro* with a cyclic adenosine monophosphate analogue and a stable progesterone (medroxy progesterone acetate) showed that decidualized eMSCs differentially secreted 12 immunomodulatory and trophic factors compared with SUSD2$^-$ fibroblasts. There was a 43-fold increase in secretion of chemokine ligand-7 (capable of attracting monocytes, lymphocytes, granulocytes, natural killer cells, and dendritic cells) and an 18-fold increase in secretion of leukemia inhibitory factor (which is a multifactorial cytokine indispensable for embryo implantation and stromal cell decidualization, as well as having roles in trophoblast attraction and differentiation). In contrast, the SUSD2$^-$ fibroblasts had only a fivefold and fourfold increase of these cytokines respectively. Therefore, eMSCs may not only have a role in the decidual response but may also function as a key immune modulator, particularly for the establishment of pregnancy given that the fetus is an allograft.

It is likely that eMSCs also have a homing capacity as they show high levels of CXCR4, a chemokine receptor for stromal-cell-derived factor 1, which is a potent chemoattractant that enables migratory activity through tissues and is upregulated in eMSCs. Furthermore, eMSCs display low levels of major histocompatibility complex class I antigens and lack major histocompatibility complex class II molecules, indicating their likely low immunogenicity. Because of these characteristics, eMSCs have been suggested as a possible source of cells for cell-based therapies and TE.

## 41.6 Culture expansion of endometrial mesenchymal stem/stromal cells toward current good manufacturing practice conditions

Adult stem/progenitor cells in the tissues are rare and account for around 1% of the total tissue. In bone marrow, MSCs account for less than 0.01% of marrow cells, which decreases even further with age [72,73]. In the endometrium, clonogenic MSCs account for around 1–5% of the stromal cells [38,59]. Owing to their limited number in tissues, MSCs need to be enriched using specific markers (see earlier) and then expanded in tissue culture to obtain sufficient numbers of cells for clinical trials. To date, there are no standard culture conditions that satisfy the growth of

MSCs from all tissue types. For clinical translation, culture of MSCs ideally requires serum and xeno-free conditions compliant with current good manufacturing practice. Using Xcelligence and cell viability assays, fibronectin was identified as a superior attachment factor for eMSCs compared with collagen, gelatin, or Cell Start XF [74]. In comparison with various serum-free media (designed for bone marrow MSC culture), an in-house serum-free medium supplemented with FGF-2 and epidermal growth factor and cultured in physiological oxygen concentration (5% $O_2$) was found optimal for eMSC growth. Under these conditions the cultured eMSCs retained their surface phenotype, cloning efficiency, and multilineage differentiation ability [74]. Substitutes for fetal calf serum include human serum, human platelet lysate, or chemically defined serum-free media [75–77]. However, serum and platelet lysates are complex products with many undefined components and significant lot-to-lot variation, hindering the development of a uniform approach to MSC expansion. Serum-free medium supplemented with chemically defined growth factors and other small molecules is the likely approach for future eMSC expansion.

Not all serum-free media support MSC growth equally [74,78,79]. For example, eMSCs grew well in Lonza-TP-SF medium, but underwent early differentiation, as demonstrated by loss of the MSC markers CD140b, CD146, and SUSD2 but not the characteristic MSC markers CD73, CD90, and CD105, highlighting the inability of classic MSC markers to distinguish MSCs from fibroblasts [74] (and see Chapter 30). Similarly, MSCs derived from umbilical cord and from adipose tissue were supported by StemPro XF medium containing 2% human serum supplementation and by xeno-free medium (RegES) respectively [75,80]. This suggests that optimal culture conditions may need to be defined for each MSC source because MSCs from different tissues have subtly different microenvironments *in situ* by which they maintain tissue homeostasis [74].

## 41.7 Tissue engineering for pelvic organ prolapse repair

Having developed a new mesh material more closely simulating vaginal tissue biomechanics and having characterized a new source of easily obtainable eMSCs, a TE construct comprising eMSCs and PA/G was developed (Figure 41.2E). *In vivo* evaluation of this TE construct comprising passage 6 eMSCs labeled with Vybrant DiO dye showed that eMSCs attached well on the PA/G mesh (Figure 41.2E). The labeled cells could be identified *in vitro* for up to 35 days and *in vivo* for at least 14 days [49]. In a wound-healing fascial defect model the PA/G–eMSCs TE construct (Figure 41.2E) was implanted subcutaneously in the dorsal region of a nude rat and studied for 3 months (Figure 41.2F). PA/G–eMSCs mesh promoted early neovascularization at 7 days, as assessed by α-SMA immunostaining and image analysis. A greater initial influx of $CD45^+$ leucocytes, predominantly proinflammatory type M1 $CCR7^+$ macrophages, was observed at 7 days in the PA/G–eMSCs mesh, but by 30 days a prohealing M2 $CD263^+$ macrophage phenotype predominated. (CD263, otherwise known as TRAIL-R3, is present on neutrophils and macrophages and is a glycophosphatidylinositol-anchored protein that lacks the cytoplasmic death domain. Thus, it is unable to induce apoptosis and serves as a negative regulator of apoptotic signaling by competing for binding of TRAIL with death receptor 5.) In comparison, the PA/G mesh alone elicited a smaller M1 response at 7 days, which was still predominant at 30 days [49]. By 90 days, significantly fewer $CD68^+$ macrophages surrounded the PA/G–eMSCs mesh filaments compared with PA/G mesh alone. The collagen deposited around the mesh was of rat origin identified by a specific anti-rat-collagen antibody. There was no difference between the total collagen production in the mesh with or without cells or in the ratio of collagen III/collagen I + III over 90 days of implantation (Figure 41.3A). Picrosirius red (Figure 41.3B) also showed similar total collagen content, but birefringence analysis showed that the quality of collagen fibrils laid down differed between the mesh alone and the eMSC-seeded mesh, which comprised well-organized physiological thin fine fibrils (Figure 41.3D) with crimping (Figure 41.3F) [81]. In comparison, the PA/G alone resulted in a greater proportion of dense bands of scar-like fibrils (Figure 41.3C) and without crimping (Figure 41.3E) [49,81]. These differences may have contributed to the biomechanically less stiff and more extensible mesh/tissue complex generated from the eMSC-seeded mesh, suggesting greater compliance and matching to the soft tissue of the vaginal wall than those without cells (Figure 41.4). Such new tissue deposition and biomechanical properties in the eMSC-seeded mesh implants are likely to be more favorable for vaginal tissue repair by allowing greater mobility [49].

The PA mesh with gelatin (10% w/v in $H_2O$) cross-linked with glutaraldehyde (0.025% w/v) was optimized for eMSCs growth and gelatin degradation *in vitro* [82]. Endometrial eMSCs differentiated into SM22-α (a marker of adult smooth muscle) and smooth muscle myosin-heavy-chain-expressing SMCs on the PA/G mesh in serum-free induction medium containing TGF-β1 and PDGF. eMSCs also differentiated into fibroblast-like cells on the PA/G mesh in 10% serum-supplemented induction media containing connective tissue growth factor (CTGF) and ascorbic acid. CTGF, also known as CCN2, is a matricellular protein of the CCN family of extracellular-matrix-associated heparin-binding proteins. The CTGF-differentiated cells produced collagen I and Tenascin-C, with total collagen production and Masson's trichrome staining indicative of new tissue formation. Tenascin-C is important in regulating cell proliferation and migration, especially during developmental differentiation and wound healing.

SMCs and fibroblasts are vital cellular components required for regenerating the fibromuscular wall of the human vagina, to provide elasticity and strength [82]. However, in the *in vivo* rat model of fascial repair the SMCs and collagen produced were of rat origin, suggesting that the human eMSCs mediated their effects by an induced paracrine mechanism [49]; this is possible given the secretome of cultured W5C5/$SUSD2^+$ eMSCs [68]. To

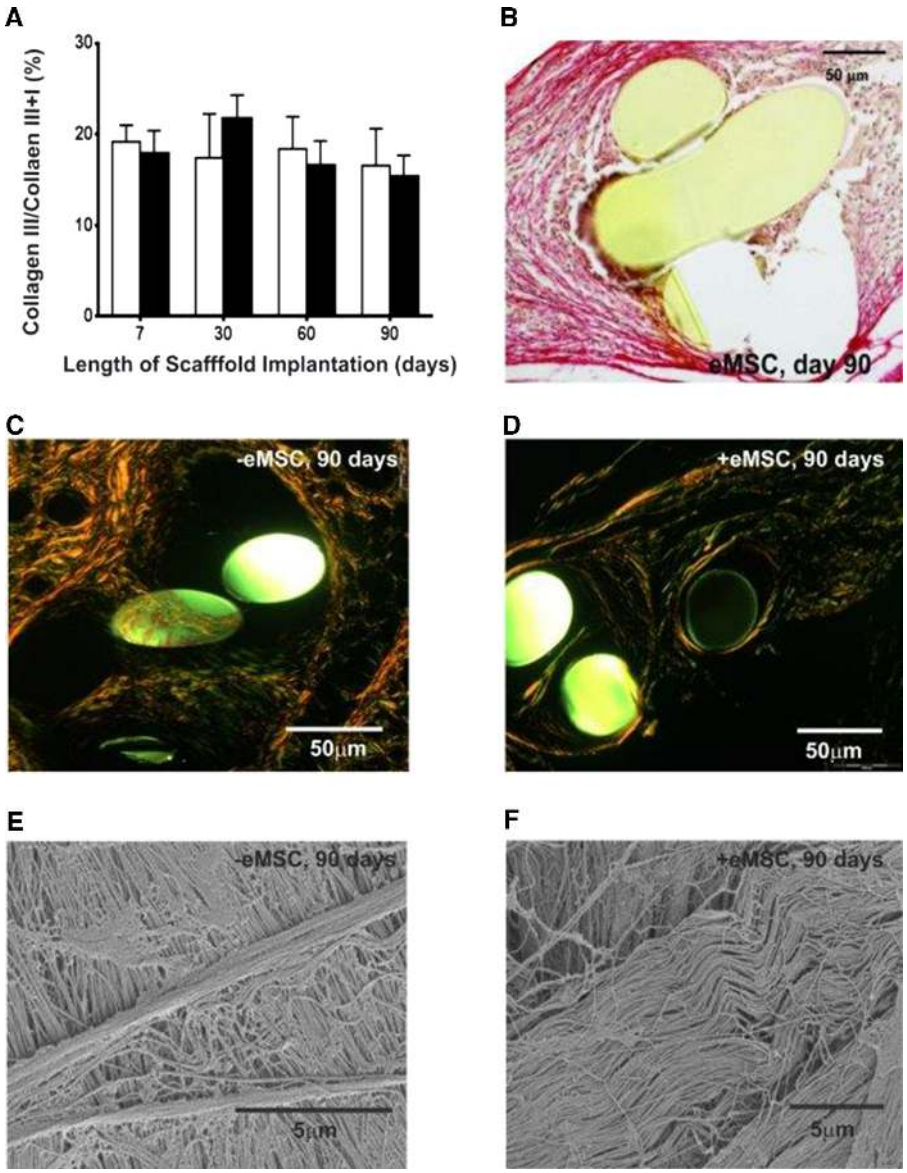



**Figure 41.3** Collagen assessment in explanted PA/G meshes with or without eMSCs. (A) PA/G meshes with (black bars) or without (white bars) eMSCs were subcutaneously implanted in the dorsal region of immunodeficient nude rats. The tissue constructs were explanted and the percentage of total collagen III within and around the meshes was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The percentage of collagen III changed little over the period of time in the two groups. Data are mean plus/minus standard error of the mean of $n = 8$ animals/group. (B) Conventional light microscopy image of Sirius-red-stained section to show collagen in explanted PA/G–eMSC mesh construct after 90 days. (C,D) Birefringence microscopy images of Sirius-red-stained sections from explanted PA/G mesh (C) and PA/G–eMSC mesh (D) after 90 days assessed using and scored according to the presence of predominantly thick (orange, red) or thin (green) birefringent collagen fibers. Rats implanted with PA/G–eMSC mesh had a greater number of thin collagen fibers, while the mesh alone had more dense and thick collagen fibers. (E,F) The explanted PA/G meshes with (F) or without (E) eMSCs were carefully extracted from the surrounding muscle and processed for scanning electron microscopy. The micrographs were taken at 1.2 kV using a Schottky field-emission variable pressure scanning electron microscope. Unseeded mesh had relatively narrow collagen bundles (up to 1.3 mm in width) with straight collagen fibrils. Seeded mesh consisted of wider collagen fiber bundles (up to 8 mm in width) with crimped configuration. (See color plate section.) *Source*: reproduced with permission from Mary Ann Liebert Inc. publishers from Ulrich *et al.* [49] *Tissue Engineering A* 2014 and Elsevier from Edwards *et al.* [81] *Acta Biomateriala* 2015.

confirm whether eMSCs are capable of producing collagen and modulating biocompatibility of the PA/G mesh *in vivo* in the vaginal environment, a preclinical large animal model of vaginal repair was required (Figure 41.2F). Currently, this new PA/G–eMSCs TE construct appears promising for POP corrective surgery.

In another study, seven different candidate materials, both biological and synthetic (AlloDerm, porcine dermis, PP, sheep

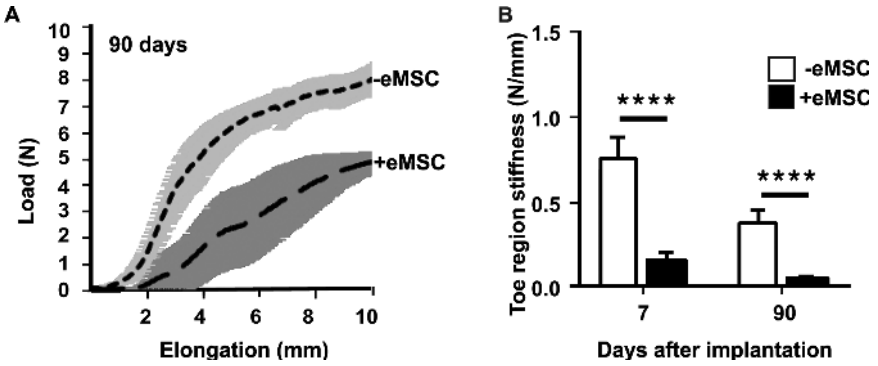



**Figure 41.4** (A) Load–elongation curves for unseeded and eMSC-seeded PA/G meshes 90 days postimplantation. Error bars are 95% confidence intervals of $n = 6$ animals/group. (B) Toe region stiffness of PA/G (white bars) and eMSC–PA/G mesh from (A). *Source*: Reproduced with permission from Mary Ann Liebert Inc. publishers from Ulrich *et al.* [49] *Tissue Engineering A* 2014.

forestomach, small intestine submucosa, acellular cadaveric dermis, and PLA scaffold), were assessed *in vitro* for cell attachment, contracture, and extracellular matrix production [83]. Alloderm is a commercially produced acellular dermal matrix. Among these materials, biodegradable PLA and SIS meshes seeded with human buccal fibroblasts showed favorable *in vitro* properties for potential clinical use. For example, these TE constructs increased new extracellular matrix production, particularly collagens I and III and elastin. Both collagen types are a vital component of the extracellular matrix, but the ratio of collagens I and III was not addressed. The biomechanical properties of the PLA mesh with or without buccal fibroblast seeding showed that strength, strain, and Young's modulus for the PLA tissue construct were similar to the biomechanical properties of native tissue from women without pelvic dysfunction [84]. Similar findings were observed when adipose-derived MSCs were used with the PLA mesh [36]. Young's modulus, also known as the tensile modulus or elastic modulus, is a measure of the stiffness of an elastic material. It is defined as the ratio of the stress (force per unit area) along an axis to the strain (ratio of deformation over initial length) along that axis. A rubber band, for example, has an extremely low Young's modulus.

In a rat model with PLA mesh seeded with or without cultured human or rat adipose-derived MSCs and implanted for 7 days, angiogenesis was only promoted in the cell-seeded mesh, as demonstrated by CD31 (platelet endothelium adhesion molecule-1, an endothelial marker) immunostaining [85]. Deposition of collagens III and I was demonstrated qualitatively by immunohistochemistry, and increased macrophage infiltration and foreign body reaction was shown by CD68 immunohistochemistry. CD68 is a glycoprotein that binds to low-density lipoprotein and is only expressed on monocytes and macrophages. There was no difference in the macrophage infiltration in the seeded and unseeded meshes, and macrophages enclosed the PLA fibers, indicating an innate immune response to the synthetic material. This 7-day study with PLA examined the short-term acute effects of fibroblasts on implanted meshes; the long-term effect of the scaffold plus cells still needs to be demonstrated. In both these studies, extracellular matrix deposition increased when fibroblasts were delivered on meshes, but there is a need for long-term *in vivo* studies to assess whether the cells make a difference in extracellular matrix production and strength and longevity of the implant. All these studies highlight that cellular therapy may significantly improve mesh biocompatibility for POP by modifying the host response to the mesh material.

### 41.7.1 A large animal preclinical model for pelvic organ proplapse

Animal models of POP are vital in providing preclinical data prior to testing any new TE approach in women. There is no perfect animal model to study POP owing to its multifactorial etiology. Humans are bipedal with a unique pelvic orientation, not recapitulated in most mammalian species. Nonhuman primates, including rhesus and squirrel monkeys, are the best animal model for POP study as they develop spontaneous prolapse similar to women [86]. These nonhuman primates are bipedal for some of the time and have similar anatomical and histological pelvic structures to humans. Their reproductive cycle and pregnancy outcome closely match those of humans. However, nonhuman primate models are limited by ethical issues, their short life span, lengthy pregnancy duration before developing POP, and prohibitive costs [86].

The sheep is a quadripedal large animal that has similar pelvic tissue anatomy, histology, size, and vaginal dimensions but a larger fetal head/maternal pelvis ratio than women [86,87]. Ewes have the same three pelvic support structures as women and their pelvises experience increased internal pressures due to their ruminant anatomy, offsetting some of the disadvantage of being quadrupedal [86]. Like humans, sheep have prolonged labors with relatively large fetuses and spontaneously develop antepartum uterovaginal prolapse at the same frequency (8–12%) as women develop chronic POP. Ovine prolapse is associated with age and pregnancy, similar to the situation in women [86]. Regional variation in the biochemical composition of the ovine vagina was discovered in a comparative study of the posterior vaginal wall in postmenopausal women and in a sheep model [88]. Total collagen and elastin tissue-associated protein content in the vaginal wall in the two species were similar. The proximal region near the cervix of the ovine vagina had significantly higher collagen and glycosaminoglycan content compared with the distal region. The proximal ovine vagina was also much

stiffer, stronger, and more elastic than more distal regions, in contrast to human vaginal tissues. There was a significantly increased percentage of type III to type I collagen (40%) in human compared with ovine vagina [88]. The increase in type III collagen in postmenopausal women correlates with their propensity to develop prolapse. The similarity between women and sheep in the biochemical composition and histology of the vaginal wall complements the similarity in the biomechanical properties and therefore makes the sheep a good preclinical model for vaginal studies involving POP (Figure 41.2F).

## 41.8 Conclusions

Endometrium is a highly regenerative tissue and it is likely that adult stem/progenitor cells are responsible for its cyclical growth, differentiation, and regression. Rare populations of perivascular human MSCs have been identified in both the functional (shed) and germinal layers of the endometrium. These eMSCs are present at all stages of the menstrual cycle, as well as in inactive and atrophic postmenopausal endometrium. eMSCs can be harvested from endometrial biopsies in an office-based procedure with minimal discomfort and without the use of anesthesia, making the endometrium an attractive, although still underrecognized, alternative source of MSCs. Importantly, eMSCs can be enriched by magnetic bead selection using the W5C5 antibody or by flow cytometry sorting using CD140b and CD146 antibodies, removing contaminating stromal cells or leucocytes not possible when using plastic adherence for MSC isolation. eMSCs are clonogenic, can self-renew *in vitro*, display multipotent differentiation into mesodermal lineages *in vitro*, and generate endometrial stromal-like tissues and vascular cells *in vivo*.

Newly fabricated polyamide–gelatin composite meshes are superior to commercially available PP meshes, promoting neovascularization, collagen deposition, and minimizing inflammatory cell infiltration. These properties were further augmented when eMSCs were integrated with PA/G mesh as a TE construct and implanted into a fascial defect in rats. The eMSCs initiated a vigorous M1 macrophage influx and promoted their differentiation to a wound-healing M2 phenotype. eMSCs promoted the deposition of physiological fibrilar, crimped nonscarring collagen fibers, significantly improving the biocompatibility and the biomechanical properties of the explanted mesh–tissue complex. These are favorable and promising profiles of the PA/G–eMSCs TE construct.

Substantial progress has been made in examining the utility of these TE constructs in a small animal model, but to take this approach to the clinic for the treatment of POP, more studies examining the mechanism of how eMSCs exert their anti-inflammatory effects are required. Finally, an autologous large animal model using the sheep will provide the necessary preclinical data for translation as a potential treatment for POP in women.

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## References

1 A. H. MacLennan, A. W. Taylor, D. H. Wilson, D. Wilson. The prevalence of pelvic floor disorders and their relationship to gender, age, parity and mode of delivery. *BJOG* 2000;**107**(12):1460–1470.
2 E. S. Lukacz, J. M. Lawrence, R. Contreras, *et al.* Parity, mode of delivery, and pelvic floor disorders. *Obstet Gynecol* 2006;**107**(6):1253–1260.
3 I. Nygaard, M. D. Barber, K. L. Burgio, *et al.* Prevalence of symptomatic pelvic floor disorders in US women. *JAMA* 2008;**300**(11):1311–1316.
4 A. L. Olsen, V. J. Smith, J. O. Bergstrom, *et al.* Epidemiology of surgically managed pelvic organ prolapse and urinary incontinence. *Obstet Gynecol* 1997;**89**(4):501–506.
5 K. B. S. Hunskaar, A. Clark, M.C. Lapita, *et al.* Epidemiology of urinary and fecal incontinence and pelvic organ prolapse. In: P. Abrams, L. Cordozo, S. Koury, A. Wein (eds), *Third International Consultation on Incontinence*, 1st edn. Health Publication: Paris, 2005.
6 A. M. Barbosa, G. Marini, F. Piculo, *et al.* Prevalence of urinary incontinence and pelvic floor muscle dysfunction in primiparae two years after cesarean section: cross-sectional study. *Sao Paulo Med J* 2013;**131**(2):95–99.
7 J. O. DeLancey. The hidden epidemic of pelvic floor dysfunction: achievable goals for improved prevention and treatment. *Am J Obstet Gynecol* 2005;**192**(5):1488–1495.
8 H. P. Dietz, V. Lanzarone. Levator trauma after vaginal delivery. *Obstet Gynecol* 2005;**106**(4):707–712.
9 J. A. Ashton-Miller, J. O. Delancey. On the biomechanics of vaginal birth and common sequelae. *Annu Rev Biomed Eng* 2009;**11**:163–176.
10 M. F. Fialkow, K. M. Newton, G. M. Lentz, N. S. Weiss. Lifetime risk of surgical management for pelvic organ prolapse or urinary incontinence. *Int Urogynecol J Pelvic Floor Dysfunct* 2008;**19**(3):437–440.
11 J. M. Wu, C. A. Matthews, M. M. Conover, *et al.* Lifetime risk of stress urinary incontinence or pelvic organ prolapse surgery. *Obstet Gynecol* 2014;**123**(6):1201–1206.
12 F. J. Smith, C. D. Holman, R. E. Moorin, N,. Tsokos. Lifetime risk of undergoing surgery for pelvic organ prolapse. *Obstet Gynecol* 2010;**116**(5):1096–1100.
13 J. E. Jelovsek, C. Maher, M. D. Barber. Pelvic organ prolapse. *Lancet* 2007;**369**(9566):1027–1038.
14 N. Howlader, A. M. Noone, M. Krapcho, *et al.* (eds). SEER Cancer Statistics Review, 1975–2010. National Cancer Institute: Bethesda, MD, 2013; http://seer.cancer.gov/archive/csr/1975_2010/results_merged/topic_lifetime_risk.pdf (accessed March 19, 2016).

15 N. Muller. What Americans understand and how they are affected by bladder control problems: highlights of recent nationwide consumer research. *Urol Nurs* 2005;**25**(2):109–115.

16 T. W. Hu, T. H. Wagner, J. D. Bentkover, *et al.* Costs of urinary incontinence and overactive bladder in the United States: a comparative study. *Urology* 2004;**63**(3):461–465.

17 US FDA, CDRH. *Urogynecologic Surgical Mesh: Update on the Safety and Effectiveness of Transvaginal Placement for Pelvic Organ Prolapse*. US Food and Drug Administration: Silver Spring, MD, 2011.

18 J. N. Nguyen, R. J. Burchette. Outcome after anterior vaginal prolapse repair: a randomized controlled trial. *Obstet Gynecol* 2008;**111**(4):891–898.

19 D. Altman, T. Väyrynen, M. E. Engh, *et al.* Anterior colporrhaphy versus transvaginal mesh for pelvic-organ prolapse. *N Engl J Med* 2011;**364**(19):1826–1836.

20 Q. Cao, Y. S. Chen, J. X. Ding, *et al.* Long-term treatment outcomes of transvaginal mesh surgery versus anterior-posterior colporrhaphy for pelvic organ prolapse. *Aust N Z J Obstet Gynaecol* 2013;**53**(1):79–85.

21 A. Bako, R. Dhar. Review of synthetic mesh-related complications in pelvic floor reconstructive surgery. *Int Urogynecol J Pelvic Floor Dysfunct* 2009;**20**(1):103–111.

22 E. J. Stanford, A. Cassidenti, M. D. Moen. Traditional native tissue versus mesh-augmented pelvic organ prolapse repairs: providing an accurate interpretation of current literature. *Int Urogynecol J* 2012;**23**(1):19–28.

23 D. R. Ellington, H. E. Richter. The role of vaginal mesh procedures in pelvic organ prolapse surgery in view of complication risk. *Obstet Gynecol Int* 2013;**2013**:356960.

24 US FDA. FDA Public Health Notification: serious complications associated with transvaginal placement of surgical mesh for pelvic organ prolapse and stress urinary incontinence. US Food and Drug Administration: Silver Spring, MD, 2008; http://www.fda.gov/MedicalDevices/Safety/AlertsandNotices/PublicHealthNotifications/ucm061976.htm (accessed March 19, 2016).

25 T. Aboushwareb, P. McKenzie, F. Wezel, *et al.* Is tissue engineering and biomaterials the future for lower urinary tract dysfunction (LUTD)/pelvic organ prolapse (POP)? *Neurourol Urodyn* 2011;**30**(5):775–782.

26 S. L. Edwards, J. A. Werkmeister, A. Rosamilia, *et al.* Characterisation of clinical and newly fabricated meshes for pelvic organ prolapse repair. *J Mech Behav Biomed Mater* 2013;**23**:53–61.

27 D. Ulrich, S. L. Edwards, J. F. White, *et al.* A preclinical evaluation of alternative synthetic biomaterials for fascial defect repair using a rat abdominal hernia model. *PLoS ONE* 2012;**7**(11):e50044.

28 J. Deprest, F. Zheng, M. Konstantinovic, *et al.* The biology behind fascial defects and the use of implants in pelvic organ prolapse repair. *Int Urogynecol J Pelvic Floor Dysfunct* 2006;**17**(Suppl 1):S16–S25.

29 M. Boennelycke, S. Gras, G. Lose. Tissue engineering as a potential alternative or adjunct to surgical reconstruction in treating pelvic organ prolapse: reply to Osman *et al. Int Urogynecol J* 2013;**24**(5):883.

30 M. H. Ho, S. Heydarkhan, D. Vernet, *et al.* Stimulating vaginal repair in rats through skeletal muscle-derived stem cells seeded on small intestinal submucosal scaffolds. *Obstet Gynecol* 2009;**114**(2 Pt 1):300–309.

31 M. Boennelycke, L. Christensen, L. F. Nielsen, *et al.* Fresh muscle fiber fragments on a scaffold in rats-a new concept in urogynecology? *Am J Obstet Gynecol* 2011;**205**(3): 235.e10-235.e14.

32 M. J. Hung, M. C. Wen, C. N. Hung, *et al.* Tissue-engineered fascia from vaginal fibroblasts for patients needing reconstructive pelvic surgery. *Int Urogynecol J* 2010;**21**(9):1085–1093.

33 A. M. Ruiz-Zapata, M. H. Kerkhof, B. Zandieh-Doulabi, *et al.* Functional characteristics of vaginal fibroblastic cells from premenopausal women with pelvic organ prolapse. *Mol Hum Reprod* 2014;**20**:1135–1143.

34 A. Kokcu, F. Yanik, M. Cetinkaya, *et al.* Histopathological evaluation of the connective tissue of the vaginal fascia and the uterine ligaments in women with and without pelvic relaxation. *Arch Gynecol Obstet* 2002;**266**(2):75–78.

35 B. Lindroos, K. Mäenpää, T. Ylikomi, *et al.* Characterisation of human dental stem cells and buccal mucosa fibroblasts. *Biochem Biophys Res Commun* 2008;**368**(2):329–335.

36 S. Roman, A. Mangera, N. I. Osman, *et al.* Developing a tissue engineered repair material for treatment of stress urinary incontinence and pelvic organ prolapse – which cell source? *Neurourol Urodyn* 2014;**33**(5):531–537.

37 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411):143–147.

38 K. E. Schwab, C. E. Gargett. Co-expression of two perivascular cell markers isolates mesenchymal stem-like cells from human endometrium. *Hum Reprod* 2007;**22**(11):2903–2911.

39 M. Crisan, S. Yap, L. Casteilla, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

40 J. Tolar, K. Le Blanc, A. Keating, *et al.* Concise review: hitting the right spot with mesenchymal stromal cells. *Stem Cells* 2010;**28**(8):1446–1455.

41 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.

42 B. Parekkadan, J. M. Milwid. Mesenchymal stem cells as therapeutics. *Annu Rev Biomed Eng* 2010;**12**:87–117.

43 A. Atala. Engineering organs. *Curr Opin Biotechnol* 2009;**20**(5):575–592.

44 L. von Bahr, I. Batsis, G. Moll, *et al.* Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells* 2012;**30**(7):1575–1578.

45 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

46 W. Wagner, S. Bork, P. Horn, *et al.* Aging and replicative senescence have related effects on human stem and progenitor cells. *PLoS ONE* 2009;**4**(6):e5846.

47 S. Sethe, A. Scutt, A. Stolzing. Aging of mesenchymal stem cells. *Ageing Res Rev* 2006;**5**(1):91–116.

48 A. N. Schuring, N. Schulte, R. Kelsch, *et al.* Characterization of endometrial mesenchymal stem-like cells obtained by endometrial biopsy during routine diagnostics. *Fertil Steril* 2011;**95**(1):423–426.

49 D. Ulrich, S. L. Edwards, K. Su, *et al.* Human endometrial mesenchymal stem cells modulate the tissue response and mechanical behavior of polyamide mesh implants for pelvic organ prolapse repair. *Tissue Eng Part A* 2014;**20**(3–4):785–798.

50 D. Ulrich, R. Muralitharan, C. E. Gargett. Toward the use of endometrial and menstrual blood mesenchymal stem cells for cell-based therapies. *Expert Opin Biol Ther* 2013;**13**(10):1387–1400.

51 R. H. R. Garry, K.A. Karthigasu, C. Burke. A re-appraisal of the morphological changes within the endometrium during menstruation: a hysteroscopic, histological and scanning electron microscopic study. *Hum Reprod* 2009;**24**:1393–1401.

52 C. E. Gargett, L. Ye. Endometrial reconstruction from stem cells. *Fertil Steril* 2012;**98**(1):11–20.

53 D. Ulrich, K. S. Tan, J. Deane, *et al*. Mesenchymal stem/stromal cells in post-menopausal endometrium. *Hum Reprod* 2014;**29**(9):1895–1905.

54 B. Ettinger, L. Bainton, D. H. Upmalis, *et al*. Comparison of endometrial growth produced by unopposed conjugated estrogens or by micronized estradiol in postmenopausal women. *Am J Obstet Gynecol* 1997;**176**:112–117.

55 R. W. Chan, K. E. Schwab, C. E. Gargett. Clonogenicity of human endometrial epithelial and stromal cells. *Biol Reprod* 2004;**70**(6):1738–1750.

56 C. E. Gargett, K. E. Schwab, R. M. Zillwood, *et al*. Isolation and culture of epithelial progenitors and mesenchymal stem cells from human endometrium. *Biol Reprod* 2009;**80**(6):1136–1145.

57 K. E. Schwab, R. W. Chan, C. E. Gargett. Putative stem cell activity of human endometrial epithelial and stromal cells during the menstrual cycle. *Fertil Steril* 2005;**84**(Suppl 2):1124–1130.

58 K. E. Schwab, P. Hutchinson, C. E. Gargett. Identification of surface markers for prospective isolation of human endometrial stromal colony-forming cells. *Hum Reprod* 2008;**23**(4):934–943.

59 H. Masuda, S. S. Anwar, H. J. Buhring, *et al*. A novel marker of human endometrial mesenchymal stem-like cells. *Cell Transplant* 2012;**21**:2201–2214.

60 K. Sivasubramaniyan, A. Harichandan, S. Schumann, *et al*. Prospective isolation of mesenchymal stem cells from human bone marrow using novel antibodies directed against Sushi domain containing 2. *Stem Cells Dev* 2013;**22**:1944–1954.

61 R. M. Ewing, P. Chu, F. Elisma, *et al*. Large-scale mapping of human protein-protein interactions by mass spectrometry. *Mol Syst Biol* 2007;**3**:89.

62 H.-J. Buhring, V. L. Battula, S. Treml, *et al*. Novel markers for the prospective isolation of human MSC. *Ann N Y Acad Sci* 2007;**1106**:262–271.

63 H. Masuda, T. Maruyama, E. Hiratsu, *et al*. Noninvasive and real-time assessment of reconstructed functional human endometrium in NOD/SCID/$\gamma_c^{null}$ immunodeficient mice. *Proc Natl Acad Sci U S A* 2007;**104**(6):1925–1930.

64 T. L. Spitzer, A. Rojas, Z. Zelenko, *et al*. Perivascular human endometrial mesenchymal stem cells express pathways relevant to self-renewal, lineage specification, and functional phenotype. *Biol Reprod* 2012;**86**(2):58.

65 M. Sobiesiak, K. Sivasubramaniyan, C. Hermann, *et al*. The mesenchymal stem cell antigen MSCA-1 is identical to tissue non-specific alkaline phosphatase. *Stem Cells Dev* 2010;**19**(5):669–677.

66 A. N. Patel, E. Park, M. Kuzman, *et al*. Multipotent menstrual blood stromal stem cells: isolation, characterization, and differentiation. *Cell Transplant* 2008;**17**(3):303–311.

67 N. H. Cho, Y. K. Park, Y. T. Kim, *et al*. Lifetime expression of stem cell markers in the uterine endometrium. *Fertil Steril* 2004;**81**(2):403–407.

68 C. A. Wells, R. Mosbergen, O. Korn, *et al*. Stemformatics: visualisation and sharing of stem cell gene expression. *Stem Cell Res* 2013;**10**(3):387–395.

69 K. Murakami, Y. H. Lee, E. S. Lucas, *et al*. Decidualization induces a secretome switch in perivascular niche cells of the human endometrium. *Endocrinology* 2014;**155**(11):4542–4553.

70 X. Meng, T. E. Ichim, J. Zhong, *et al*. Endometrial regenerative cells: a novel stem cell population. *J Transl Med* 2007;**5**:57.

71 X. Wang, J. Dai. Concise review: isoforms of OCT4 contribute to the confusing diversity in stem cell biology. *Stem Cells* 2010;**28**(5):885–893.

72 A. Stolzing, E. Jones, D. McGonagle, A. Scutt. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech Ageing Dev* 2008;**129**(3):163–173.

73 A. I. Caplan. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009;**217**(2):318–324.

74 G. Rajaraman, J. White, K. S. Tan, *et al*. Optimization and scale-up culture of human endometrial multipotent mesenchymal stromal cells: potential for clinical application. *Tissue Eng Part C Methods* 2013;**19**(1):80–92.

75 I. Hartmann, T. Hollweck, S. Haffner, *et al*. Umbilical cord tissue-derived mesenchymal stem cells grow best under GMP-compliant culture conditions and maintain their phenotypic and functional properties. *J Immunol Methods* 2010;**363**(1):80–89.

76 J. C. Chachques, J. Herreros, J. Trainini, *et al*. Autologous human serum for cell culture avoids the implantation of cardioverter-defibrillators in cellular cardiomyoplasty. *Int J Cardiol* 2004;**95**(Suppl 1):S29–S33.

77 A. Aldahmash, M. Haack-Sorensen, M. Al-Nbaheen, *et al*. Human serum is as efficient as fetal bovine serum in supporting proliferation and differentiation of human multipotent stromal (mesenchymal) stem cells *in vitro* and *in vivo*. *Stem Cell Rev* 2011;**7**(4):860–868.

78 N. Fekete, M. T. Rojewski, R. Lotfi, H. Schrezenmeier. Essential components for *ex vivo* proliferation of mesenchymal stromal cells. *Tissue Eng Part C Methods* 2014;**20**:129–139.

79 B. Lindroos, S. Boucher, L. Chase, *et al*. Serum-free, xeno-free culture media maintain the proliferation rate and multipotentiality of adipose stem cells *in vitro*. *Cytotherapy* 2009;**11**(7):958–972.

80 K. Rajala, B. Lindroos, S. M. Hussein, *et al*. A defined and xeno-free culture method enabling the establishment of clinical-grade human embryonic, induced pluripotent and adipose stem cells. *PLoS ONE* 2010;**5**(4):e10246.

81 S. L. Edwards, D. Ulrich, J. F. White, *et al*. Temporal changes in the biomechanical properties of endometrial mesenchymal stem cell seeded scaffolds in a rat model. *Acta Biomater* 2015;**13**:286–294.

82 K. Su, S. L. Edwards, K. S. Tan, *et al*. Induction of endometrial mesenchymal stem cells into tissue-forming cells suitable for fascial repair. *Acta Biomater* 2014;**10**:5012–5020.

83 A. Mangera, A. J. Bullock, S. Roman, *et al*. Comparison of candidate scaffolds for tissue engineering for stress urinary incontinence and pelvic organ prolapse repair. *BJU Int* 2013;**112**(5):674–685.

84 L. Lei, Y. Song, R. Chen. Biomechanical properties of prolapsed vaginal tissue in pre- and postmenopausal women. *Int Urogynecol J Pelvic Floor Dysfunct* 2007;**18**(6):603–607.

85 S. Roman Regueros, M. Albersen, S. Manodoro, *et al*. Acute *in vivo* response to an alternative implant for urogynecology. *BioMed Res Int* 2014;**2014**:853610.

86 B. M. Couri, A. T. Lenis, A. Borazjani, *et al*. Animal models of female pelvic organ prolapse: lessons learned. *Expert Review Obstet Gynecol* 2012;**7**(3):249–260.

87 S. D. Abramowitch, A. Feola, Z. Jallah, P. A. Moalli. Tissue mechanics, animal models, and pelvic organ prolapse: a review. *Eur J Obstet Gynecol Reprod Biol* 2009;**144**(Suppl 1):S146–S158.

88 D. Ulrich, S. L. Edwards, V. Letouzey, *et al*. Regional variation in tissue composition and biomechanical properties of postmenopausal ovine and human vagina. *PLoS ONE* 2014;**9**(8):e104972.

CHAPTER 42

# Closed automated large-scale bioreactors for manufacturing mesenchymal stromal cells for clinical use

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## 42.1 Introduction

Over 600 clinical trials using mesenchymal stromal cells (MSCs) or mesenchymal stem cells have been listed on ClinicalTrials.gov if you type into the search box either mesenchymal stromal cells or mesenchymal stem cells. This site is run by the United States National Library of Medicine (NLM) at the National Institutes of Health. Obtaining MSCs for clinical trials is a cumbersome, time-consuming, and laborious process when done manually (see Chapters 3–5 and 7). In addition, the prolonged period of *ex vivo* expansion includes the risk of the cells becoming microbially infected. Thus, we attempted to develop an automated closed system that could be scaled up for manufacturing MSCs for clinical trials. We chose term placenta obtained at elective cesarean section, since this provides a large amount of biomaterial rich in these cells that would otherwise have been discarded. The rate of elective cesarean sections performed at term in Australia is between 30 and 50%, making access feasible. We had shown previously that adult human MSCs derived from term placenta were similar to those derived from adult human bone marrow in terms of cell surface phenotype, ability to suppress T cell alloproliferation in *vitro*, and with similar differentiation ability *in vitro* to chondrogenic and osteogenic lineages [1,2]. They did not differentiate to adipogenesis to the same degree as that of marrow-derived MSCs [1], and the gene expression profiles differed to some extent [3]. However, we have found them safe when injected intravenously in people with idiopathic pulmonary fibrosis in a phase I clinical trial [4] and capable of regenerating new tendon tissue when injected directly into the Achilles tendon in people with chronic Achilles tendinopathy refractory to surgery or exercise programs (Young *et al.*, unpublished results, and see Chapter 47).

## 42.2 Design of a semi-automated closed-system bioreactor capable of manufacturing mesenchymal stromal cells for clinical use

We employed a noncontact paddle blender for semi-automated processing of term placentas. In these blenders the tissue was placed into a sterile sample bag that was then subjected to a crushing action by alternating compression of the contents by mechanical paddles. An enzyme digest solution was added to the bag prior to blending in order to facilitate the release of cells from tissue fragments [5].

Various seeding and culture formats were explored. These included static dishes/flasks, static tubes, shake tubes, shake flasks, and spinner flasks. The most consistent performance was ultimately achieved by overnight seeding under

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static conditions in a minimum amount of complete medium. After 6–7 days culture in the same medium as above, microcarriers were collected into sterile tubes, washed with Hanks' balanced salt solution, and the cells released by enzymatic treatment. Microcarriers screened for placental MSC culture included Cytodex 1, Cytodex 3, CultiSpher-S, Hillex II, MicroHex, Cytopore 1, and Cytopore 2. CulltiSphere-S was selected for subsequent experiments. Following further washing in Hanks' balanced salt solution to remove enzymes, cells were reseeded on to fresh microcarriers. Wave-type bioreactor microcarrier culture CultiSpher-S microcarriers were introduced to 2 L CultiBags or Cellbags. Cells were then similarly introduced in a minimal volume of medium and the bag gently rocked several times in order to distribute the cells amongst the microcarriers. Following incubation of cells with microcarriers for 18 h, culture medium was collected by decanting [5].

The blender-based approach was capable of processing an entire placenta in 1.5–4 h. We found that neither our blender-based approach to isolation, nor subsequent cultivation on CultiSpher-S microcarriers altered the MSC phenotype relative to standard flask expansion. There was no indication that cultivation of human placental MSCs on CultiSpher-S was detrimental to the final cell product. Although simple, our isolation and culture methods were highly effective. We found that cultivation of MSCs from a single 500 g placenta over four serial passages would have the potential to produce more than 7000 doses per placenta in a scaled-up version of this bioreactor system.

## 42.3 A commercially available closed-system bioreactor for manufacturing mesenchymal stromal cells for clinical use

Terumo BCT sell a machine called the Quantum™ specifically for manufacturing cells, including MSCs, for clinical purposes. The Quantum system's 3D, functionally closed bioreactor contains about 11 500 hollow fibers with a surface area of 2.1 $m^2$, which is equivalent to the surface area of 120 T175 flasks. The bioreactor's design and small footprint of 0.3 $m^2$ footprint allow more effective use of laboratory space. The Quantum system can replace the need for an incubator, a biosafety cabinet, and a clean room, which are typically required for flask-based culture methods and which are expensive to operate. It is a functionally closed system with temperature and gas control.

The large surface area is designed to maximize adherent cell expansion in a minimal amount of space. The bioreactor provides a defined and stable geometry that allows for the control and management of the cell culture environment. The closed environment of the bioreactor remains intact during the cell culture process, removing open events and hands-on tasks associated with manual flask-based culture methods. The Quantum system uses a number of predefined tasks that can be customized to replicate the manual cell culture process for a range of adherent cell types, including mesenchymal stromal cells. These tasks include automated cell seeding, reagent addition, feeding, and harvest. Continuous temperature control, cell feeding, and waste removal provide process control over the cell culture environment. A direct gas connection allows operators to control the cell culture environment, enabling any gas blend connection to the Quantum system.

An internal investigation by Terumo BCT demonstrated that hypoxic and normoxic conditions had no significant difference on cell yield, viability, phenotypic, morphologic, or differentiation potential. The Quantum system is controlled by a touchscreen user interface, which allows operators to monitor and adjust cell culture process parameters. The Quantum system provides reporting, remote alarms, user authentication, and power failure recovery features, providing more control over the cell culture process, aiding in current good manufacturing practice and associated compliance.

The system includes four primary phases. First, the Quantum system is primed as the nutrients, metabolites, and gases diffuse across the fibers in the bioreactor. Second, cells are loaded into the bioreactor. Third, media exchange begins with the continuous control of temperature, cell feeding, and waste removal. Finally, cells are harvested in as little as 20 min.

The National Institutes of Health analyzed bone marrow stromal cells (BMSCs) from the Quantum system to determine if they retained their differentiation potential. It was found, first, that cell yields in all test cases were higher when cells were grown in the Quantum system compared with cell factories. Second, BMSCs grown in the Quantum system were found to have similar phenotypes when compared with BMSCs grown in cell factories. Lastly, BMSCs from both the Quantum system and cell factories were able to differentiate into adipocytes.

Lechanteur *et al.* compared the Quantum device with traditional clinical culture system in flasks [6]. They found that the Quantum device reproducibly produced therapeutic MSC doses that fulfilled the International Society for Cell Therapy release criteria, were sterile, devoid of mycoplasma and endotoxin, had normal karyotypes, and demonstrated immunosuppressive and differentiation capacities *in vitro*. Cells grew faster in the bioreactor than in flasks during passage P1 (doubling time 40 h compared with 56 h in flasks) and P2 expansions but not during the primary expansion phase (P0). Seeding $20 \times 10^6$ thawed P2-preselected cells on the device allowed harvesting of $110–276 \times 10^6$ MSCs after a 7-day expansion. Seeding $50 \times 10^6$ cells resulted in $291–334 \times 10^6$ harvested MSCs. If MSCs become part of mainstream medicine, it appears that such a system will be infinitely cheaper and safer to use than the manual methods currently still used at many institutions for manufacturing MSCs.

## References


1 Barlow S, Brooke G, Chatterjee K, *et al*. Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stromal cells. *Stem Cells and Development* 2008;**17**:1095–1107.

2 Jones BL, Brooke G, Atkinson K, McTaggart SJ. Immunosuppression by placental indoleamine 2,3-dioxygenase: a role for mesenchymal stem cells. *Placenta* 2007;**28**:1174–1181.

3 Matigian N, Brooke G, Zaibak F, *et al*. Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern. *Genomics Data* 2015;**3**,70–74.

4 Chambers DC, Enever D, Ilic N, *et al*. A phase 1b study of placenta-derived stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2014;**19**:1013–1018.

5 Timmins N, Kiel G, Heazlewood C, *et al*. Closed system isolation and scalable expansion of human placental mesenchymal stem cells. *Biotechnology and Bioengineering* 2012;**109**:1817–1826.

6 Lechanteur C, Baila S, Janssens ME, *et al*. Large-scale clinical expansion of mesenchymal stem cells in the GMP-compliant, closed automated Quantum® cell expansion system: comparison with expansion in traditional T-flasks. *Journal of Stem Cell Research & Therapy* 2014;**4**:222. doi: 10.4172/2157-7633.10002223.

SECTION V

# GMP manufacturing of mesenchymal stromal cells for clinical use

CHAPTER 43

# Current good manufacturing practice for the isolation and *ex vivo* expansion of mesenchymal stromal cells derived from term human placenta for use in clinical trials

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## 43.1 Source of mesenchymal stromal cells for use in clinical trials

Bone marrow has been the traditional source of mesenchymal stromal cells (MSCs), but, because MSCs are components of blood vessel walls, they can be isolated from any vascularized organ or tissue [1]. Ideally, sources that are easily accessible and contain large amounts of tissue are the most useful. MSCs have been isolated from blood and bone marrow, adipose tissue, amniotic fluid, umbilical cord blood, umbilical cord including Wharton's jelly and perivascular tissue, first trimester fetal liver, and second trimester fetal liver, lung, bone marrow, and pancreas, as well as from term placenta and amniotic and chorionic placental membranes. Gestational sources can be collected at delivery or from prenatal diagnostic specimens such as amniotic fluid. The latter can be sourced by the minimally invasive technique of amniocentesis, or obtained peripartum from otherwise discarded tissue.

We compared human term placenta-derived MSCs with human bone-marrow-derived MSCs and could find no significant difference in their ability to suppress T cell alloproliferation *in vitro* [2], cell surface phenotype, cytokine receptor display, or mesodermal lineage differentiation to osteogenic and cartilage lineages *in vitro* [3]. Placenta-derived MSCs, however, consistently showed less differentiation ability to adipogenesis *in vitro* compared with marrow MSCs [3]. Additionally, gene array expression has revealed differences between human MSCs derived from adult bone marrow and those derived from term placenta [4]. Whether these differences affect functional outcome when the placental MSCs are administered for a specific indication is currently unknown, although the results of trials in which our placenta-derived MSCs have been used have been encouraging (see later).

Since obtaining 50 mL of bone marrow from a normal volunteer donor is a painful procedure, and because we had on campus

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a maternity hospital in which 10 000 babies were born each year, 40% of them by elective cesarean section (C section) at term, we elected to use term placenta-derived MSCs for our MSC clinical trials program.

## 43.2 Inclusion criteria for mothers wishing to donate their term placenta for isolation and expansion of mesenchymal stromal cells for use in clinical trials approved by a human research ethics committee

All mothers giving birth by elective lower segment C section at the Mater Mothers Hospital, Brisbane, Australia, who had signed informed consent and who did not fulfill any of the exclusion criteria listed in Section 43.3 were included in the phase 1 clinical trials described later in this chapter.

## 43.3 Exclusion criteria for mothers wishing to donate their term placenta for isolation and expansion of mesenchymal stromal cells for use in clinical trials approved by a human research ethics committee

We imposed strict exclusion criteria on the selection of mothers who agreed to donate their term placenta. First, we included all the exclusion criteria used by the Australian Red Cross for excluding blood donors. Second, because term placenta includes tissues of fetal origin (the amniotic and chorionic membranes) as well as tissues of maternal origin (the decidual placenta), we added all the exclusion criteria of the Australian Cord Blood Registry (AusCord) not listed by the Australian Red Cross.

Donors of placenta were required to undergo a blood test to determine their serological status of the following: the presence of hepatitis B surface antigen, and antibodies against hepatitis A, hepatitis B, hepatitis C, human immunodeficiency virus (HIV) I/II, human T cell leukemia virus I/II, Epstein–Barr virus, cytomegalovirus (CMV), herpes simplex virus, varicella-zoster virus, syphilis, and toxoplasmosis.

Maternal donors had to be seronegative for all of the serological tests with the exception of CMV, Epstein–Barr virus, herpes simplex virus, varicella-zoster virus, and toxoplasmosis. However, MSCs manufactured from CMV-seropositive mothers were not given to clinical trial subjects who were CMV seronegative.

In addition, the following exclusion criteria were applied.

- A previous or current serious blood disorder.
- A previous or current cancer, except adequately treated basal cell carcinomas of the skin or adequately treated carcinoma *in situ* of the uterine cervix.
- Known carriers for, or mothers with partners who were considered to have, risk factors for any infectious disease transmissible by blood product transfusion, including hepatitis B and C, HIV, human T cell leukemia virus I/II, and syphilis.
- A serious infections during pregnancy.
- Residence in the United Kingdom during the period between January 1, 1980, and December 31, 1996, for a period of more than 6 months or if visits on several occasions during those years added up to a total of more than 6 months.
- Age less than 18 years old or more than 45 years old.
- Less than 36 weeks' gestation at the time of delivery or with a multiple pregnancy.
- Refusal to have their blood tested for sexually transmitted diseases or the serology tests described earlier.
- Use of intravenous drugs not prescribed by a doctor.
- Prior treatment with clotting factors such as factor VIII or factor IX.
- An inherited blood disorder, such as Fanconi anemia, Blackfan–Diamond syndrome, pyruvate kinase deficiency, thalassemia, hereditary spherocytosis, or hereditary ellliptocytosis.
- Multiple sclerosis, myasthenia gravis, or polymyositis.
- Receipt of injections of human pituitary hormone for infertility injections or human growth hormones for short stature before 1986.
- A previous allogeneic organ or tissue transplant (e.g., a corneal transplant, a skin graft, a hematopoietic stem cell transplant, a kidney, liver, lung, heart, or pancreas transplant) or a dura mater patch.
- Creutzfeldt Jakob disease (or its variant), familial insomnia or Gerstmann Staussler Scheinker disease, or Chagas' disease.
- Sexual contact with a person known to have been exposed to HIV.
- Sexual contact with a male who had sexual contact with another male in the preceding 5 years.
- Sexual contact with a person who had had sexual contact with a male or female sex worker.
- Shared needles or syringes for drug abuse, or had
  - a blood products transfusion or an intra-uterine transfusion within the preceding 12 months;
  - injury with a used needle/syringe;
  - a blood/body fluid splash to eyes, mouth, nose, or broken skin;
  - jaundice or hepatitis or contact with a person who had had malaria, Ross River fever, dengue fever, leptospirosis, Q fever, brucellosis, or worked in an abattoir.
- Serious heart disease, including cardiomyopathy, myocarditis, pericarditis, or rheumatic heart disease.
- On immunosuppressive therapy during pregnancy (such as corticosteroids for autoimmune disorders, including ankylosing spondylitis, multisystem autoimmune disease, systemic lupus erythematosus, polymyositis, or rheumatoid arthritis).
- A history within the last 6 months of persistent fevers, night sweats, diarrhea, or lymphadenopathy.
- Infectious mononucleosis or pancreatitis within 1 month.
- Active genital herpes, chicken pox, herpes zoster, meningitis, amebic dysentery, shigella or salmonella infections, food

poisoning, giardia infection, viral infection of unknown cause, Coxsackie virus, or yersinia infection within the last 2 weeks.
- Antibiotic therapy for any named or other illness within the last 2 weeks.
- Refusal to sign the Placenta Donation Declaration form.

## 43.4 Mesenchymal stromal cell manufacturing

### 43.4.1 The good manufacturing process facility

This must be a facility approved by the Therapeutic Goods of Australia for cell manufacturing and cell banking. While the current federal law does not mandate the use of such a facility for phase 1 clinical trials, we elected to use such a facility in order to maximize the safety and reproducibility of the MSC product, as well as maximally protecting the manufacturing scientists and the reputation of the institution.

Significant preparation is required before starting the manufacturing procedure. Gowning including double gloving is required (Figures 43.1 and 43.2).

The anteroom and the clean room must have been prepared before the gowned scientists and quality control personnel enter the facility.

Environmental monitoring takes place before and during the manufacturing procedure (Figure 43.3).



**Figure 43.1** Gowning prior to entering the clean room.

Finger swabs taken from each person entering the anteroom or the clean room were an important part of this monitoring (Figure 43.4).

### 43.4.2 Quality control and quality assurance

Quality control (QC) and quality assurance (QA) are pivotal to current good manufacturing practice (GMP). We wrapped our MSC manufacturing procedure in a tight series of standard operating procedures and protocols. Any deviation from one of these documents raised an immediate warning that was investigated and dealt with as quickly as possible. Our QC/QA program contained over 2000 documents and required a 0.5 full-time equivalent person to build it and manage it.

### 43.4.3 Isolating and expanding mesenchymal stromal cells from human term placenta

The MSCs isolated and manufactured in *ex vivo* expansion cultures were obtained from the term placenta of women undergoing elective C section delivery at term. Using good clinical practice, good laboratory practice, and GMP, the placental tissue was received in the clean room of the GMP facility (day 0). The umbilical cord was drained. The whole placenta was then cut into small pieces using scalpels and scissors (Figure 43.5).

Dissected pieces of placental tissue were washed in Hanks' buffered salt solution to remove excess blood, transferred to tubes and digested for 3 h at 37 °C with collagenase I and DNase I. The tissue was then passed through a cell strainer and washed. Red cells were removed using a red cell lysis agent and the remaining cells were resuspended and cultured in Dulbecco's minimal essential medium/10% fetal calf serum. After 24 h, nonadherent cells were washed off and the remaining cells maintained in culture at 37 °C in 5% $CO_2$ and 20% $O_2$. At 80–90% confluence the cells were passaged. This occurred approximately once weekly (Figures 43.6 and 43.7).

At the end of each passage the cells were harvested; all cells from a given passage were pooled and were frozen using a controlled-rate freezer to −90 °C, when they were cryopreserved in the vapor phase of liquid nitrogen. A sample from each pooled passage was sent for the tests indicated in Section 43.3.4. In addition, a small cryovial was attached to each bag or vial intended for use in a clinical trial. Three weeks prior to the planned MSC administration date, this vial was thawed and tested, again as indicated in Section 43.3.4. MSCs obtained by this procedure expressed CD29, CD44, CD73, CD90, CD105, CD166, and major histocompatibility complex (MHC) class I and did not express CD34, CD45, CD14, or MHC class II. An overview of the manufacturing process is shown in Figure 43.8.

### 43.4.4 Testing performed on mesenchymal stromal cells manufactured for clinical use

Tests performed on each passage of manufactured MSCs at the time of cryopreservation and criteria for their acceptability were as follows:




**Figure 43.2** The anteroom and clean room.




**Figure 43.3** Environmental monitoring.

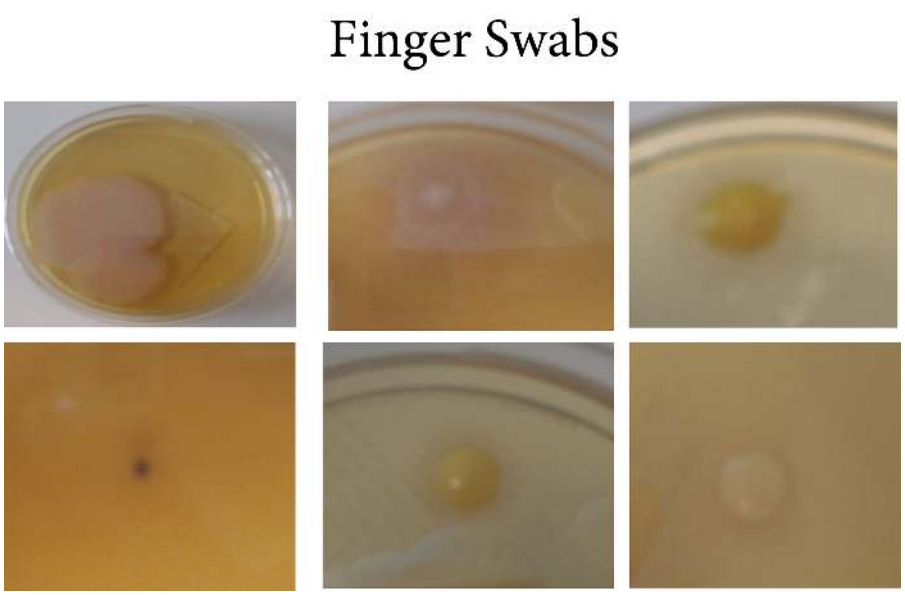



**Figure 43.4** Microbial cultures of finger swabs from the production scientists.

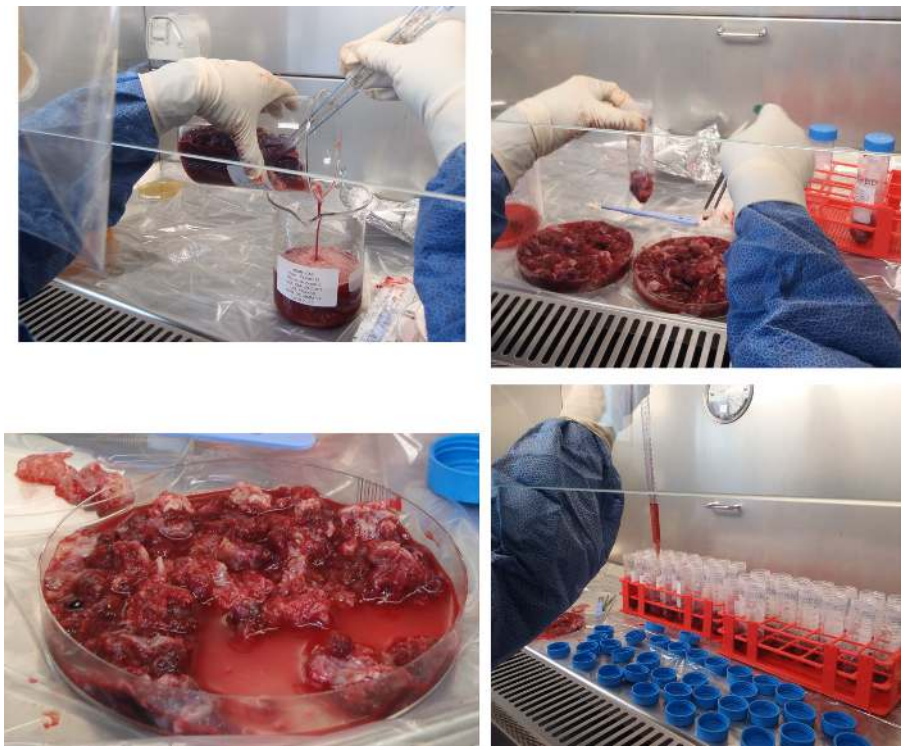


**Figure 43.5** Dissection of human placenta using scissors and scalpels prior to enzyme digestion.

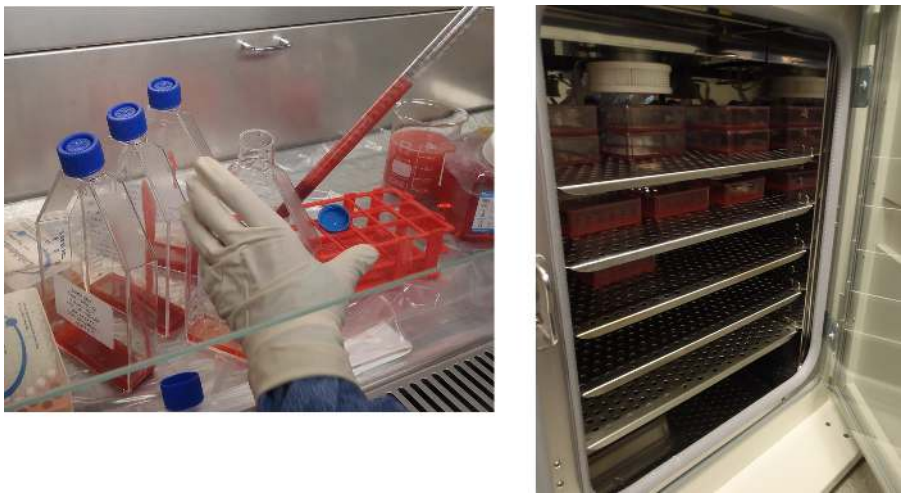


**Figure 43.6** *Ex vivo* expansion at 37 °C in 5% $CO_2$ and 20% $O_2$.

**1** Viability tested by Trypan blue dye exclusion >70%
**2** Purity as defined by >90% $CD73^+/CD105^+$ and CD45 <1%
**3** Sterility on 14-day microbial culture
**4** Mycoplasma test negative
**5** Endotoxin level <2 EU/mL
**6** 16s rRNA test (for microbial elements) negative
**7** Karyotype normal on cytogenetic testing.

Tests performed on the cryovial accompanying each cryovial or cryobag intended for use in a clinical trial and thawed 3 weeks before the planned administration of the corresponding MSCs included the following:

**1** Viability by Trypan blue dye exclusion >70%
**2** Sterility on 14-day microbial culture
**3** Mycoplasma test negative
**4** 16s rRNA test (for microbial elements) negative.

## 43.5 Phase 1 trials using placenta-derived mesenchymal stromal cells

Our laboratory generated enough placental MSCs for three phase 1 clinical trials using two maternal donors who were unrelated to, and MHC-unmatched with, the MSC recipients.




**Figure 43.7** Human placental MSCs in a tissue culture flask at day 3 of expansion culture.

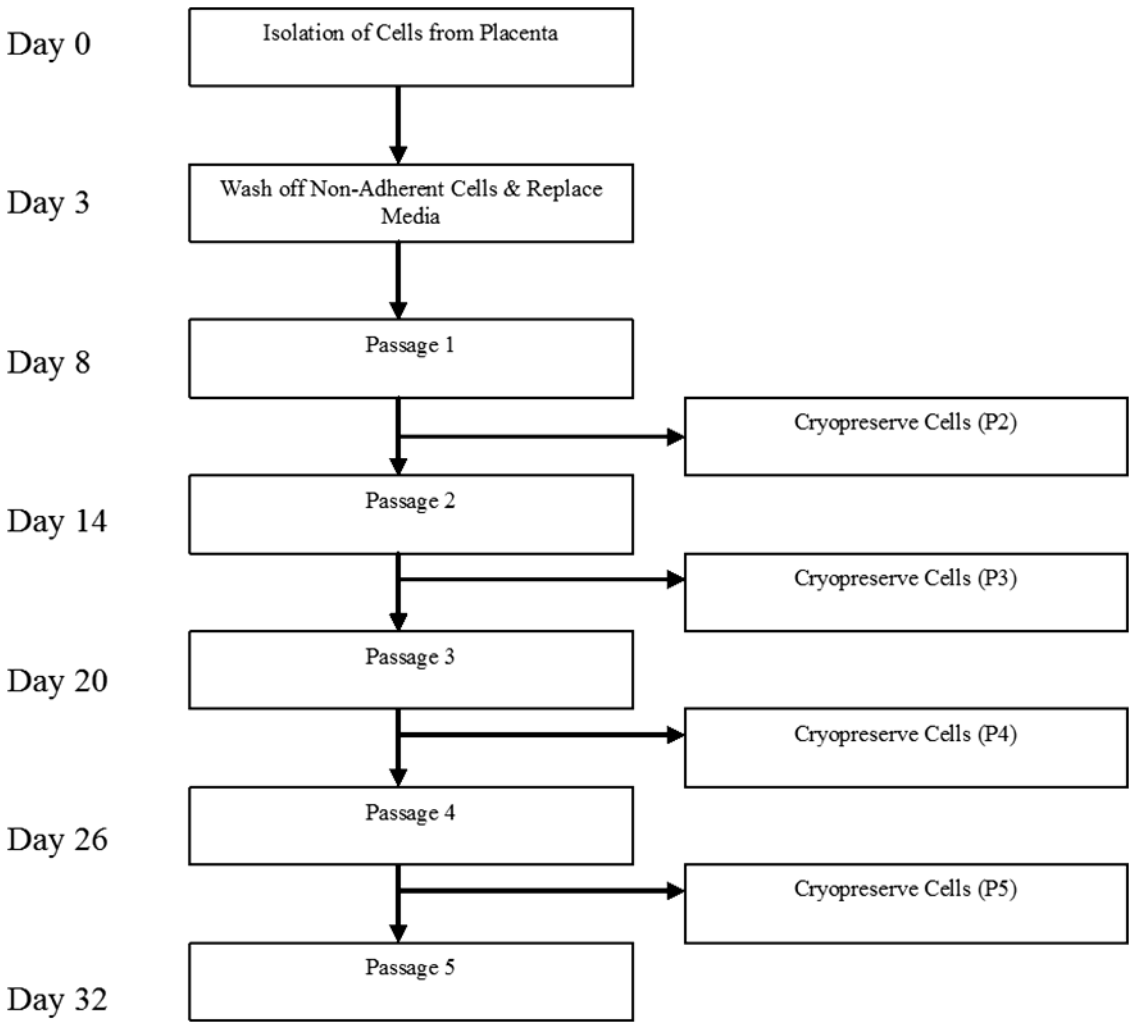



**Figure 43.8** An overview of the manufacturing process for isolating MSCs from human term placenta.

Our first trial was a phase 1 multicenter open-label dose-escalation study of unrelated, MHC-unmatched placenta-derived MSCs in recipients of unrelated umbilical cord blood hematopoietic stem cell transplants. No adverse events related to the MSCs were reported [5].

The second trial was a phase 1 trial of unrelated MHC-unmatched placenta-derived MSCs administered intravenously in patients with idiopathic pulmonary fibrosis. This disease affects middle-aged individuals and there is no effective treatment other than lung transplantation. It is usually fatal after 3–5 years. The primary objective of this trial was to establish the feasibility and safety of intravenous infusions of placental MSCs in people with this serious lung disease. No serious adverse events related to the MSCs were noted [6].

The third trial was a phase 1 trial of placenta-derived MSCs administered intratendinously in recipients with treatment-refractory Achilles tendinopathy. Two of the three planned dose-escalation cohorts with MSCs injected directly into the tendon under ultrasound guidance have been performed. No serious adverse events related to the MSCs were noted and a remarkable improvement in symptoms and exercise performance was noted (Young *et al.*, unpublished results; and see Chapter 47).

## References

1 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**:301–313.

2 Jones BL, Brooke G, Atkinson K, McTaggart SJ. Immunosuppression by placental indoleamine 2,3-dioxygenase: a role for mesenchymal stem cells. *Placenta* 2007;**28**:1174–1181.

3 Barlow S, Brooke G, Chatterjee K, *et al.* Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stromal cells. *Stem Cells Dev* 2008;**17**:1095–1107.

4 Matigian N, Brooke G, Zaibak F, *et al.* Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern. *Genomics Data* 2015;**3**:70–74.

5 Brooke G, Rossetti T, Pelekanos R, *et al.* Manufacturing of human placenta-derived mesenchymal stem cells for clinical trials. *Br J Haem* 2008;**144**:571–579.

6 Chambers DC, Enever D, Ilic N, *et al.* A phase 1b study of placenta-derived stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2014;**19**:1013–1018.

CHAPTER 44

# A comparison of high-tier regulatory documents pertaining to biologic drugs including mesenchymal stromal cells in Australia, Europe, and the USA using a manual documentary analysis

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## 44.1 Introduction

The development of a wide range of regulatory standards applicable to the production and use of tissues, cells (including mesenchymal stromal cells) and other biologics (or biologicals) as advanced therapies indicates considerable interest in governmental regulation of these products. In this chapter, high-tier documents within the Australian, European, and US biologic drug regulatory environments have been analyzed and compared using qualitative methodology. Cohort 1 of the selected 18 high-tier regulatory documents from the European Medicine Agency (EMA), the US Food and Drug Administration (FDA). and the Australian Therapeutic Goods Administration (TGA) were subject to a manual documentary analysis. These documents are consistent with the legal requirements for the manufacturing and use of biologic drugs in humans and fall into six different categories. Manual analysis included a terminology search. The occurrence, frequency, and interchangeable use of different terms and phrases were recorded in the manual documentary analysis. Despite obvious differences, manual documentary analysis revealed general consistency in the use of terminology across frameworks analyzed. Phrase search frequencies showed less uniformity than the search of terms. The EMA framework's documents referred to "medicinal products" and "marketing authorisation(s)," the FDA documents discussed "drug(s)" or "biologic(s)," and the TGA documents referred to "biological(s)." Although high-tier documents often utilized different terminology, they shared concepts and themes. Documents originating from the same source have more conjunction in their terminology, although they belong to different frameworks (e.g., good clinical practice requirements based on the Declaration of Helsinki, 1964). Automated (software-based) documentary analysis can also be obtained for conceptual and relational analysis.

## 44.2 Background

The recent development of a wide range of regulatory standards applicable to the production and use of tissues, cells, and other biologics (or biologicals) – as new therapies – has stimulated considerable interest in the regulation of biologic drugs (including, but not limited to, human cell and tissue therapies) as part of the vast array of biopharmaceutical agents available or becoming available. Mesenchymal stromal cells are an example of this. Governmental regulation of biological therapeutic agents has been widely developed by mature regulatory agencies such as the Australian framework administered by the TGA, the European Union's EMA levied framework, and the US framework imposed by the FDA [1].

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A significant number of documents are currently available in these regulatory frameworks in the form of standards, guidelines, recommendations, or regulations [2]. Conditions of product manufacturing and release have been established and outlined in a spectrum of documents from high-tier regulatory documents down to procedural documents. However, one of the major issues facing manufacturers, clinical practitioners, patients, patient advocacy groups, investors, and the general public is confusion over terminology at various levels and in types of documents applicable to specific products (e.g., stem cells versus progenitor cells versus biologics versus biologicals) [2–6]. Additional misconceptions can be attributed to the unclear level of regulatory oversight required, different levels of scrutiny (e.g., control of an entire manufacturing facility versus control of a manufacturing process), differences in availability of specific formal written procedures (e.g., some regulatory documents are deemed overly prescriptive or overly generic), and different product testing and release requirements [2–6]. Nonetheless, complex regulations are meant to be adhered to at all times and in every aspect of the manufacturing of biologic drugs (including, but not limited to, human cell and tissue therapies).

For the purpose of this chapter, all the aforementioned products, including mesenchymal stromal cells, will be referred to as biologic drugs, although other terminology may be used in the literature [3]; however, it should be noted that consensus has not been reached entirely on the use of the term "drug" [4], the regulatory oversight required [5], and established industry models [6]. An overview of the regulatory documents available on the websites of these three regulatory agencies is shown in Table 44.1.

The intention of this chapter is to compare and contrast the content and terminology in high-tier regulatory documents related to biologic drugs in Australia, Europe, and the USA, where these products are also referred to as biologicals, Advanced Therapy Medicinal Products (ATMPs), and biologics respectively. Since stem cells and their progeny are "biologics," these regulations are of major importance to all those attempting to translate stem cells or progenitor cells into clinical trials and clinical practice.

To accomplish this, an assessment of specific documents was made. The scope of the study included regulations of biologic drugs in Australia, Europe, and USA, while the objective was to derive data shared between the regulatory frameworks in order to make them usable in a harmonization process. Exploiting the shared terminology and mutually recognized scope or aim of the regulations was easiest to define via documentary analysis of high-tier documents in each regulatory scheme. It was hypothesized that there are similarities between high-tier regulatory documents related to biologic drugs in Australia, Europe, and the USA, although the terminology and focus of these regulatory frameworks differ to some extent. It was also anticipated that high-tier regulatory documents could be taken as a central representation of the regulatory frameworks from which they were drawn. The overall aim of this chapter is to assess the use of a qualitative methodology in this highly specialized area of regulatory science.

## 44.3 Definitions used by the Australian Therapeutic Goods Administration, the European Medicine Agency, and the US Food and Drug Administration for "biologicals"

The following definitions were used by the three regulatory agencies. The TGA regulatory documents refer to biologicals as [7]:

> Essentially a biological comprises, contains or is derived from human cells or human tissues . . . and is used to treat or prevent disease, ailment, defect or injury; or to diagnose the condition of a person; or to influence, inhibit or modify a physiological process in persons; or to test the susceptibility of persons to a disease or ailment; or to replace or modify parts of the anatomy in persons.

The official FDA definition of biological products or biologics can be summarized as "any virus, therapeutic serum, toxin, antitoxin or analogous product applicable to the prevention, treatment or cure of diseases or injuries of man," while the FDA Consumer Information page states [8]:

> Biological products include a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues, and recombinant therapeutic proteins. Biologics can be composed of sugars, proteins, or nucleic acids or complex combinations of these substances, or may be living entities such as cells and tissues. Biologics are isolated from a variety of natural sources – humans, animals, or microorganisms – and may be produced by biotechnology methods and other cutting-edge technologies. Gene-based and cellular biologics, for example, often are at the forefront of biomedical research, and may be used to treat a variety of medical conditions for which no other treatments are available.

The lengthy official definition (codified in 21 CFR 600.3) vaguely defines biologics on the basis of analogies (i.e., products similar to viruses, serums, toxins and antitoxins) [9]. This definition avoids terms and concepts in use for generations (i.e., proteins, antibodies, genes, microbes, cells, viruses, and DNA/RNA). In practice, biologics include "a wide range of products such as vaccines, blood and blood components, allergenics, somatic cells, gene therapy, tissues and recombinant therapeutic proteins" [9].

The European Parliament Regulation 1394/2007 (amending directive 2001/83/EC and Regulation 726/2004) identifies an ATMP as [10]:

> . . . a medicinal product as defined in Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the Community code relating to medicinal products for human use (the Directive), as amended to reflect new innovative therapeutic products. Specifically, an ATMP is a biological medicinal product which is either: - a gene therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC; a somatic cell therapy medicinal product as defined in Part IV of Annex I to Directive 2001/83/EC; or a tissue engineered product as defined in Article 2 1 (b) of the ATMP Regulation (1394/2007/EC).

**Table 44.1** Overview of regulatory documents available on the websites of the TGA, the EMA, and the FDA.

*Australia: overview of the TGA website structure and content* [a]

- Home page
- Safety information
- Consumers
- Health professionals
- About the TGA
- News room
- Industry
  - Regulation basics
    - How therapeutic goods are regulated in Australia
    - Australian Register of Therapeutic Goods
    - Legislation & legislative instruments
    - Advertising therapeutic goods
    - Labelling & packaging
    - Import & export
    - Clinical trials*
    - Cosmetics
    - Regulatory reforms
    - Safety information for industry
  - Over-the-counter medicines
  - Medical devices & IVDs
  - Prescription Medicines
  - Complementary medicines
  - Other therapeutic goods
  - Manufacturing therapeutic goods
  - Scheduling medicines & poisons
- Blood, tissues & biologicals
- Blood & tissues regulation
- Blood & tissues regulation basics
  - Regulation of blood
  - Regulation of tissues
    - Current situation
    - The future
- Standards, guidelines & publications
  - Australian code of GMP for human blood and tissues
- Current standards applicable to TGA-licensed HPC manufacturers
  - Enforcements guidelines
- Guidelines for preparation of Technical Master Files for blood, blood components and haematopoietic progenitor cells (HPC)
- Forms for the blood & tissues industry
  - Blood & tissues forms
  - Related forms
- Biologicals regulation
  - Biologicals regulation basics
- Regulatory framework for biologicals
  - TGA biologicals framework newsletter
    - 2010
    - 2009
- Standards, guidelines & publications
  - Biologicals
  - Enforcement guidelines
- Forms for the biologicals industry
  - Biologicals forms (no content)
  - Related forms
    - Client details form
    - Request to transfer the sponsorship of an ARTG entry
      - Form for relinquishing company
      - Form for accepting company

*The EU: overview of the EMA website references to ATMPs Regulations as of December 10, 2011* [a]

**Regulation of medicines**

Human medicines

This section of the website provides information for companies and individuals involved in developing and marketing medicines for use in the EU.

**Table 44.1** (*Continued*)

For further information on EU legislation and procedures for the regulation of medicines, see also Volumes 1–4, 9, and 10 of the rules governing medicinal products in the EU.*

*__Eudralex__

The body of EU legislation in the pharmaceutical sector is compiled in Volume 1 and Volume 5 of the publication *The rules governing medicinal products in the European Union*.

Volume 1 – EU pharmaceutical legislation for medicinal products for human use

Volume 5 – EU pharmaceutical legislation for medicinal products for veterinary use

The basic legislation is supported by a series of guidelines that are also published in the following volumes of *The rules governing medicinal products in the European Union*:

Volume 2 – Notice to applicants and regulatory guidelines for medicinal products for human use

Volume 3 – Scientific guidelines for medicinal products for human use

Volume 4 – Guidelines for good manufacturing practices for medicinal products for human and veterinary use

Volume 6 – Notice to applicants and regulatory guidelines for medicinal products for veterinary use

Volume 7 – Scientific guidelines for medicinal products for veterinary use

Volume 8 – Maximum residue limits

Volume 9 – Guidelines for pharmacovigilance for medicinal products for human and veterinary use

Volume 10 – Guidelines for clinical trial

Medicinal products for pediatric use, orphan, herbal medicinal products, and advanced therapies are governed by specific rules.

**Advanced therapies**

Advanced-therapy medicinal products (ATMPs) are medicines for human use that are based on gene therapy, somatic-cell therapy or tissue engineering. They offer groundbreaking new opportunities for the treatment of disease and injury.

The regulatory framework for ATMPs is established in Regulation (EC) No 1394/2007.

These pages provide information for applicants on:
- the classification of ATMP marketing-authorization applications;
- certification of the quality of ATMPs;
- nonclinical data submitted by small and medium-sized enterprises (SMEs) developing ATMPs.

**Support to companies**

The Innovation Task Force (ITF) provides a forum for informal dialogue between the EMA and companies or individuals in the early stages of the medicine development process.

In addition, the agency can provide support to companies through:
- the SME office; scientific advice and protocol assistance; orphan designation.
- EU regulation on advanced therapies ATMP classification (includes scientific recommendations), certification procedure for SMEs, marketing-authorization application submission, scientific guidelines, risk management, interested parties guidance.

**Procedural advice**

Procedural advice on the provision of scientific recommendation on classification of ATMPs in accordance with article 17 of regulation (EC) No 1394/2007.

**Other information**

EU regulation on advanced therapies

ATMP classification

Certification procedure for SMEs

Marketing-authorization application submission

Scientific guidelines

Risk management

Interested parties

Guidance

*USA: overview of the FDA regulations applicable to biologic drugs since 2004* [21]

21 CFR 1271.10(a) sets out the criteria that form the foundation of our tiered, risk-based approach to regulating HCT/Ps. HCT/Ps that meet all of these criteria are subject only to regulation under section 361 of the PHS Act. These HCT/Ps are subject to the regulations in Part 1271, and no pre-market approval is required. HCT/Ps that do not meet all of the criteria in 21 CFR 1271.10(a) are regulated as drugs, devices, and/or biological products. These HCT/Ps are subject to the regulations specific to drugs, biological products, or medical devices, in addition to applicable sections of Part 1271. Bone (including demineralized bone), Ligaments, Tendons, Fascia, Cartilage, Ocular Tissue (Corneas and Sclera), Skin, Arteries and Veins (except umbilical cord

**Table 44.1** (*Continued*)

veins), Pericardium, Amniotic membrane (when used alone, without added cells for ocular repair), Dura mater, Heart valve allografts, Semen, Oocytes, Embryos and Hematopoietic stem/progenitor cells derived from peripheral and cord blood.
On 25 May 2004, the FDA disseminated regulations requiring most cell and tissue donors to be tested and screened for relevant communicable diseases (donor-eligibility final rule, 69 FR 29786). On 18 November 2004, the FDA issued regulations that require establishments that manufacture human cell and tissue products (HCT/Ps) to comply with Current Good Tissue Practices (CGTP), which include proper handling, processing, labelling, and record-keeping procedures. At the same time, FDA also published an interim final rule to revise certain regulations regarding the screening and testing of HCT/P donors and related labelling (interim final rule, 70 FR29949). The FDA took this action in response to comments from interested persons regarding the impracticability of complying with certain regulations as they affect particular HCT/Ps. The CGTP and other regulations are contained in 21 CFR Part 1271, along with provisions relating to establishment registration. These regulations apply to HCT/Ps recovered on or after the rule's effective date. HCT/Ps that were recovered before the effective date of the new rules are subject to 21 CFR 1270, and subparts A and B of Part 1271, as appropriate. In addition, 21 CFR Part 1271 subparts A, B, C, F, 21 CFR 1271.150(c) and 21 CFR 1271.155 of subpart D apply to reproductive HCT/Ps. The new Part 1271 is made up of six subparts: general provisions pertaining to the scope and purpose of Part 1271, as well as definitions; registration and listing procedures; provisions for the screening and testing of donors to determine their eligibility; current Good Tissue Practice (cGTP) requirements; certain labelling and reporting requirements; inspection and enforcement provisions. Those HCT/Ps that do not meet all 21 CFR 1271.10(a) criteria and are regulated as drugs, devices or biological products are covered under separate compliance programs:

- Blood and blood products are covered under CP 7342.001 Inspection of Licensed and Unlicensed Blood Banks, Brokers, Reference Laboratories, and Contractors; and CP 7342.002 Inspection of Source Plasma Establishments;
- HCT/Ps that do not meet all 21 CFR 1271.10(a) criteria and are regulated as Medical Devices are covered under CP 7382.845 Inspection of Medical Device Manufacturers;
- HCT/Ps that do not meet all 21 CFR 1271.10 (a) criteria i.e. autologous, allogeneic or xenogeneic cells whose biological characteristics have been altered (i.e. propagated, pharmacologically treated) *and ex vivo* and gene therapy products are regulated as biological drugs and are covered under CP 7345.848 Inspection of Biological Drug Products;
- HCT/Ps recovered before 25 May 2005 and regulated under 21 CFR 1270 and subparts A and B of Part 1271 are covered under CP 7341.002A Inspection of Tissue Establishments.

The above HCT/Ps are regulated solely under section 361of the PHS Act and the regulations in 21 CFR Part 1271 if they meet all of the following criteria: minimally manipulated; intended for a homologous use only as reflected by the labelling, advertising, or other indications of the manufacturer's objective intent; not combined with another article, (except for water, crystalloids, or a sterilizing, preserving, or storage agent, if the addition of the agent does not raise new clinical safety concerns with respect to the HCT/P); and either:

- Do not have a systemic effect and are not dependent upon the metabolic activity of living cells for the primary function; OR
- Have a systemic effect or are dependent upon the metabolic activity of the other cells for the primary function, AND:
- Are for autologous use; Are for allogeneic use in a first or second-degree relative; OR Are for reproductive use.

HCT/P establishments must follow CGTP requirements to prevent the introduction, transmission, or spread of communicable diseases by ensuring that the HCT/Ps do not contain communicable disease agents, that they are not contaminated, and that they do not become contaminated during manufacturing. The following are Core CGTP requirements as referenced in 21 CFR 1271.150(b):

- Requirements relating to facilities (21 CFR 1271.190(a) and (b))
- Requirements relating to environmental controls (21 CFR 1271.195(a))
- Requirements relating to equipment (21 CFR 1271.200(a))
- Requirements relating to supplies and reagents (21 CFR 1271.210(a) and (b))
- Requirements relating to recovery (21 CFR 1271.215)
- Requirements relating to processing and process controls (21 CFR 1271.220)
- Requirements relating to labeling controls (21 CFR 1271.250(a) and (b))
- Requirements relating to storage (21 CFR 1271.260(a) - (d))
- Requirements relating to receipt, pre-distribution shipment, and distribution of an HCT/P (21 CFR 1271.265(a)–(d)).
- Requirements relating to donor eligibility determinations, donor screening, and donor testing (sections 1271.50, 1271.75, 1271.80 and 1271.85).

ATMP, advanced therapy medicinal product; ARTG, Australian Register of Therapeutic Goods; CFR, Code of Federal Regulation; GMP, good manufacturing practice; HCT/P, human cell and tissue products.
[a] https://www.tga.gov.au/.
[b] Current EMA website available at http://www.ema.europa.eu/ema/.

## 44.4 Complexity of the area

Taking newly created biomedical discoveries from the bench to the bedside has proven to be a complex, highly challenging, and costly exercise [11]. It has been defined as translational research and recognized by researchers, funding agencies, and more recently by regulatory agencies as an imminent need [11]. Success in translational research requires not only highly trained experts and complex skill sets within a team, but also an understanding of the application of data in different sets of regulatory principles [12]. The independent development of regulatory authorities in Australia, Europe, and the USA has led to differences in the regional approach to biopharmaceutical manufacturing. Some of these were favorable for the conduct of early clinical trials with a minimal requirement for supporting data. Other developments have been affected by external factors or have expanded their scope through the years. Recent efforts have been made to harmonize the three frameworks, mostly in the area of active pharmaceutical ingredient inspections [13], and are expected to be followed by other joint programs.

Although therapeutic agents derived from biological sources have been subject to regulatory oversight for some time (e.g., monoclonal antibodies), biologic products used in transplantation procedures such as cells and tissues have historically been exempted from such oversight [14]. The unique source of the "active ingredients" renders cell and tissue therapies difficult for assessment by the traditional regulatory approaches, which have been geared to support traditional pharmaceutical manufacturing practice [15]. It has been recognized that therapeutic claims for biologics need to be supported by strong scientific evidence [16], as well as their manufacturing being governed by comprehensive regulatory principles. New considerations have led the existing regulatory agencies to propound new regulatory approaches for the sector of biologic drugs or biopharmaceuticals that "has become one of the most research-intensive sectors with great potential for delivering innovative human medicines in the future" [17].

The biopharmaceutical industry is defined as business activities related to the development of new biopharmaceutical drugs and medicines [18]. Defining the term "biopharmaceutical" for the industry's stakeholders, including the regulatory agencies, has proven to be a difficult task. Regulatory definitions of biopharmaceuticals are generally based on a broad biotechnology definition but often use other terms with their own intricate definitions (e.g., "biologicals" in Australia, ATMPs in Europe, and "biologics" in the USA) [9]. Documentary analysis is a useful methodology to clarify the complexity of biologic drugs regulations, as part of the complex regulatory arena of biopharmaceuticals [19].

Regulation of cell and tissue products is closely linked not only to sensitive areas of public health policy and funding, but also to the development of novel disciplines such as regulatory science [18,20]. This positions regulators in a challenging position as they attempt to reconcile their roles as independent assessors with public health needs and perceptions [18,20]. The historical background of the development of regulatory agencies of interest has been explored previously [21], and regional differences were illustrated by observations pertinent to their clinical trial requirements.[1] These differences inevitably contribute to the complexity of the regulations applicable to the development of biologic drugs.

## 44.5 Analysis of documents

Documents and written material used in this chapter provide a way of gaining access to sets of processes that cannot be observed or documented otherwise and are not easily described in verbal or other reconstructions [19]. Documents used were readily available on the websites of the three regulatory agencies used for comparison. They were complete, well constructed, and clearly presented. The main tasks in the manual document analysis were (1) the selection process of documents to be analyzed – necessary because of the vastness of the material available – and (2) thorough reading and analysis of selected documents. The type of sampling used in this research was theoretical or purposive sampling of high-tier regulatory documents that are, for the purpose of this chapter, taken as the central representation of the regulatory frameworks from which they were drawn. The sampling approach is outlined in Table 44.2, indicating the inclusion and exclusion criteria. An extensive list of the documents analyzed is presented in Table 44.3.

Manual analysis of selected documents identified main terms and phrases in individual documents. While undertaking data analysis, important consideration was given to selectivity and the perspective of the research, in order to maintain focus in the process of documentary analysis. This related back to asking the question of what the documents were expected to provide in an ontological and epistemological sense. Ontology in this chapter is used to indicate a rigorous and exhaustive organization of the knowledge domain of regulatory science. Epistemology in this chapter is used to indicate a philosophical approach to the nature of regulatory science, including its presuppositions, its foundation, its extent, and its validity. The process of analysis involved several steps. First, the context was established while the complexity and historical background were explored for each of the sets of regulatory principles analyzed. Second, extensive preparation for analysis and selection of documents was obtained. Third, selected documents were copied from the official regulatory agencies' websites and examined. Since the intent of this chapter is to focus predominantly on the literal wording and terminology as an examination of the documents' focus and intent, a manual method (Table 44.4) was devised to record these.

An overview of the TGA website structure and content is presented in Table 44.1. It includes only the new Biologicals Framework as established in July 2011 from which relevant documents were selected.[2] In terms of the EU regulatory requirements, selection of the documents was not straightforward owing to the complexity of national and EMA regulations (Table 44.1). It is acknowledged by the EMA that new technologies, therapies, and medicines are emerging in the form of regenerative medicine and more personalized medical treatments.[3] The lack of an EU-wide regulatory framework in the past led to divergent national approaches in Europe that hindered patient access to products; as

[1] http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp; http://www.ema.europa.eu/ema/index.jsp?curl=pages/about_us/general/general_content_000235.jsp&murl=menus/about_us/about_us.jsp∣ http://www.fda.gov; https://www.tga.gov.au/regulation-basics; https://www.tga.gov.au/clinical-trials; https://www.tga.gov.au/clinical-trials-glance; https://www.tga.gov.au/publication/note-guidance-clinical-safety-data-management-definitions-and-standards-expedited-reporting.

[2] https://www.tga.gov.au/regulation-basics; https://www.tga.gov.au/clinical-trials; https://www.tga.gov.au/clinical-trials-glance; https://www.tga.gov.au/publication/note-guidance-clinical-safety-data-management-definitions-and-standards-expedited-reporting.

[3] http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp; http://www.ema.europa.eu/ema/index.jsp?curl=pages/about_us/general/general_content_000235.jsp&murl=menus/about_us/about_us.jsp&mid.

**Table 44.2** Inclusion and exclusion criteria for selection of documents.

| Parameters | Inclusion criteria | Exclusion criteria |
|---|---|---|
| Location | EU | Regions other than EU, USA, and Australia |
| | USA | |
| | Australia | |
| Language | English | Documents not written in English |
| Type of the document | High-tier legal and regulatory domain | Documents other than high-tier legal and regulatory domain |
| Purpose of the document | Legal requirements for manufacture and use of biologic drugs for human use, including definitions and scope | Documents concerned with issues other than legal requirements for manufacture, GMP, and GCP applicable to medicinal products and biologic drugs for human use |
| | Good manufacturing practice (GMP) for manufacture of medicinal products and biologic drugs for human use | |
| | Good clinical practice (GCP) for use of medicinal products and biologic drugs in clinical trials involving humans | |
| Scope of the document | Medicinal products, biologics, biologicals, and ATMPs for human use | Documents concerned with other than medicinal products, biologics, biologicals, and ATMPs for human use |
| Time frame | Most recent documents published from 2000 (inclusive) | Documents published prior to 2000[a] |

[a] Only one document was exempt as it was originally published in 1996 (see Table 44.4).

a result, the central procedure agency was established.[4] As a further illustration of the complexity and vastness of written material available in the area of biologic drug regulations, an overview of the relevant FDA regulations since 2004 [22–24] is also presented briefly in Table 44.1. Some of these documents were selected for the analysis, based on the inclusion and exclusion criteria, while provision was made in relation to so-called nonbinding documents in the form of guidances (see the Disclaimer footnote in Table 44.3).

In terms of sampling, it was important to decide what was significant in the context of the research, its theoretical and empirical impacts, and how it related to the broader universe and the ongoing development in the field [25–29].

The data presented in Tables 44.5 and 44.6 were derived from the manual analysis, using the PDF search feature to assess the documents in Cohort 1 (see Table 44.3). Rather than treating the data as an end product, it was classified and subsequently parts or "slices" were treated as unfinished resources for further use [19]. Manual analysis included a terminology search with a list of 10 specific terms (Table 44.5) and 16 phrases (Table 44.6). The frequencies of terms and phrases in the documents are also illustrated in Figures 44.1 and 44.2.

The frequency of terms presented in Table 44.5 revealed some consistency across regions in the use of "manufacturing" (58, 71, and 113 occurrences in EMA, FDA, and TGA documents respectively) and "human" (137, 55, and 89 rates respectively). The terms "drug(s)," "cell(s)," and "biologic/biological(s)" were also to some extent used across the entire spectrum of documents analyzed.

More frequent and specific use of "medicinal" was observed in the EMA documents (a total of 1092 occurrences in three documents), while "pharmaceutical" was very sporadically utilized in any documents other than in EMA DOC1 (54 occurrences). The term "therapy(ies)" was not mentioned in any of the FDA documents and was seldom used in the TGA documents (13 times in total), while it was mentioned 141 times in EMA DOC3, likely due to the definition by the EMA of cell therapies as ATMPs.

While the most frequent words used in the EMA documents were "product(s)" along with "medicinal," the term "drug(s)" was consistently mentioned within all FDA documents (84, 34, and 36 times) but seldom mentioned in the other two frameworks (a total of five occasions). The TGA documents featured "biologic/biological(s)" most consistently, while "cell(s)" was most utilized in a document from the FDA framework (78 occurrences in FDA DOC3) and the TGA framework (100 occurrences in TGA DOC1). Considering the relevant definitions, these search terms were expected to be dominant[5] [22–24].

The definitions of cell therapies by each of the regulatory agencies are as follows.

[4] http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp; http://www.ema.europa.eu/ema/index.jsp?curl=pages/about_us/general/general_content_000235.jsp&murl=menus/about_us/about_us.jsp&mid.

[5] http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp; http://www.ema.europa.eu/ema/index.jsp?curl=pages/about_us/general/general_content_000235.jsp&murl=menus/about_us/about_us.jsp∣ http://www.fda.gov; https://www.tga.gov.au/regulation-basics; https://www.tga.gov.au/clinical-trials; https://www.tga.gov.au/clinical-trials-glance; https://www.tga.gov.au/publication/note-guidance-clinical-safety-data-management-definitions-and-standards-expedited-reporting.

**Table 44.3** List of documents analyzed.

| Group of documents | | Document title | Document version | Year of issue/ revised | Current document |
|---|---|---|---|---|---|
| **Cohort 1** | | | | | |
| *European Medicines Agency ( EMA)* | | | | | |
| Document code | EMA DOC1 | Directive 2001/83/EC | n/a | 2001 | Yes[a] |
| | EMA DOC2 | Regulation 726/2004 | n/a | 2004 | Yes[a] |
| | EMA DOC3 | Regulation 1394/2007 | n/a | 2007 | Yes |
| *Food and Drug Administration ( FDA)* [b] | | | | | |
| Document code | FDA DOC1 | 21CFR312 | n/a | 2011 | Yes |
| | FDA DOC2 | 21CFR600 Biological Products: General | n/a | 2011 | Yes |
| | FDA DOC3 | 21CFR1271 Human Cells, Tissues, and Cellular and Tissue-based Products | n/a | 2011 | Yes |
| *Therapeutic Goods Administration ( TGA)* | | | | | |
| Document code | TGA DOC1 | Australian Regulatory Guidelines for Biologicals Part 1 | 1.0 | 2011 | Yes |
| | TGA DOC2 | Australian Regulatory Guidelines for Biologicals Part 2 | 1.0 | 2011 | Yes |
| | TGA DOC3 | Australian Regulatory Guidelines for Biologicals Part 3 | 1.0 | 2011 | Yes |
| **Cohort 2** | | | | | |
| *Good manufacturing practice (1)* | | | | | |
| Document code | GMP DOC1 | Directive 2003/94/EC (EMA) | n/a | 2003 | Yes |
| | GMP DOC2 | Australian Code of Good Manufacturing Practice – Human Blood and Tissues (TGA) | n/a | 2000 | Yes[c] |
| | GMP DOC3 | Australian Code of Good Manufacturing Practice for Medicinal Products (TGA) | n/a | 2002 | Yes |
| *Good manufacturing practice (2)* | | | | | |
| Document code | GMP DOC4 | 21CFR211 Current Good Manufacturing Practice for Finished Pharmaceuticals | n/a | 2011 | Yes |
| | GMP DOC5 | Guide to Good Manufacturing Practice for medicinal Products -Part I (PIC/S) | n/a | 2009 | Yes |
| | GMP DOC6 | Guide to Good Manufacturing Practice for medicinal Products - Part II (PIC/S) | n/a | 2009 | Yes |
| *Good clinical practice* | | | | | |
| Document code | GCP DOC1 | Directive 2003/20/EC (EMA) | n/a | 2001 | Yes |
| | GCP DOC2 | Guidance for Industry E6 Good Clinical Practice (FDA) | n/a | 1996[d] | Yes |
| | GCP DOC3 | Note for Guidance on Good Clinical Practice (CPMP/ICH/135/95) Annotated (TGA) | n/a | 2000 | Yes |

PIC/S: Pharmaceutical Inspection Convention (PIC) and Pharmaceutical Inspection Co-operation Scheme; n/a: not applicable.
[a] Subsequently amended.
[b] Disclaimer: major, specific, and updated FDA guidances relating to key subsets of biologics were not included in Cohort 1 and 2 documents. Although the basic FDA regulations have not been changed much since the expansion of biologic drugs, including vaccines, the guidances have really become more like interim regulations. Owing to the research sampling criteria, guidances (including the key manufacturing guidances for biologics that the FDA does enforce in a way similar to that for regulations) have not been analyzed here. These documents can be found at http://www.fda.gov/BiologicsBloodVaccines/GuidanceComplianceRegulatoryInformation/default.htm.
[c] In process of being amended.
[d] Document was exempt from the timeframe.

**Table 44.4** Literal wording, form, and sequence of manual recording in the documents.

| Manual document analysis | Considerations | Questions raised | Result |
|---|---|---|---|
| Stage 1:<br>Selection of documents (including comparison with other frameworks/areas) | Regulatory frameworks (by region) – areas of regulatory frameworks | What to analyze? | List of documents to be analyzed |
| Stage 2:<br>Reading and "interpretation" | Purpose and aim of documents.<br>Understanding of the content | How to analyze? | Thorough understanding of aim, scope and depth of documents.<br>List of terms and phrases to search |
| Stage 3:<br>Search by key word and/or by phrase | Generalizability of terms. Terminology specific to regional regulations | Common (shared) features? | Frequencies of specific terminology and/or phrases |
| | Consistency and quality assurance<br>Presenting results in an adequate manner | Reliability of results? | Tables and diagrams outlining results of the manual analysis |

- Somatic cell therapy and gene therapy related definitions and considerations by the FDA (http://www.fda.gov) [24]:

> Somatic cell therapy is the administration to humans of autologous, allogeneic, or xenogeneic living cells which have been manipulated or processed ex vivo. Manufacture of products for somatic cell therapy involves the ex vivo propagation, expansion, selection (see: "A Proposed Approach to the Regulation of Cellular and Tissue-based Products," Feb. 28, 1997, (62 FR 9721)), or pharmacologic treatment of cells, or other alteration of their biological characteristics. Such cellular products might also be used for diagnostic or preventive purposes. Recently, various innovative therapies involving the introduction of somatic cells into humans have been used or proposed. For the purpose of this Guidance, the term somatic cell therapy refers to the administration to humans of autologous, allogeneic, or xenogeneic living non-germline cells, other than transfusable blood products, for therapeutic, diagnostic, or preventive purposes. . . . Examples of somatic cell therapies include implantation of cells as an in vivo source of a molecular species such as an enzyme, cytokine or coagulation factor; infusion of activated lymphoid cells such as lymphokine-activated killer cells and tumor-infiltrating lymphocytes (addressed in a separate Points to Consider document: see below); and implantation of manipulated cell populations, such as hepatocytes, myoblasts, or pancreatic islet cells, intended to perform a complex biological function. Because of the complexities of potential interactions with the cells and other constituents, additional components should be considered as part of the final biological product for purposes of preclinical evaluation.

**Table 44.5** Terminology search: frequency of terms in documents (cohort 1).

| Terminology search | Frequency in the documents | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | EMA | | | FDA | | | TGA | | |
| | DOC1 2001/83 | DOC2 726/2004 | DOC3 1394/2007 | DOC1 21CFR 312 | DOC2 21CFR 600 | DOC3 21CFR 1271 | DOC1 Aclr1 P1 | DOC2 Aclr1 P2 | DOC3 Aclr1 P3 |
| Approve(d)/approval(s) | 16 | 5 | 0 | 14 | 9 | 15 | 24[a] | 30 | 55[a] |
| Biologic/biological(s) | 0 | 2 | 2 | 6 | 89 | 17 | 377 | 219 | 67 |
| Cell(s) | 1 | 2 | 46 | 1 | 5 | 78 | 100 | 6 | 1 |
| Drug(s) | 1 | 0 | 0 | 84 | 34 | 36 | 1 | 3 | 0 |
| Human | 0 | 84 | 53 | 11 | 6 | 38 | 83 | 1 | 5 |
| Manufacturing | 41 | 8 | 9 | 2 | 48 | 21 | 56 | 57 | 0 |
| Medicinal | 615 | 329 | 148 | 0 | 0 | 0 | 2 | 0 | 0 |
| Product(s) | 727 | 350 | 227 | 14 | 197 | 49 | 144 | 21 | 6 |
| Pharmaceutical | 54 | 8 | 4 | 1 | 1 | 0 | 3 | 0 | 1 |
| Therapy/ies | 4 | 7 | 141 | 0 | 0 | 0 | 13 | 0 | 0 |

[a] Includes term "unapproved."

**Table 44.6** Terminology search: frequency of phrases in documents (cohort 1).

| Phrases search | Frequency in the documents | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | EMA | | | FDA | | | TGA | | |
| | DOC1 2001/83 | DOC2 726/2004 | DOC3 1394/2007 | DOC1 21CFR 312 | DOC2 21CFR 600 | DOC3 21CFR 1271 | DOC1 Aclr1 P1 | DOC2 Aclr1 P2 | DOC3 Aclr1 P3 |
| Clinical investigation | 0 | 0 | 0 | 13 | 1 | 0 | 0 | 0 | 0 |
| Clinical trial | 21 | 8 | 4 | 10 | 2 | 0 | 11 | 2 | 41 |
| Class/classification | 7 | 0 | 0 | 0 | 0 | 0 | 5 | 16 | 0 |
| Human cell(s) and tissue(s) | 0 | 0 | 1 | 0 | 0 | 1 | 0 | 0 | 0 |
| Human cells | 0 | 0 | 8 | 0 | 0 | 11 | 12 | 0 | 0 |
| Investigational new drug(s) | 0 | 0 | 0 | 16 | 2 | 0 | 0 | 0 | 0 |
| Investigational drug(s) | 0 | 0 | 0 | 14 | 0 | 0 | 0 | 0 | 0 |
| Marketing authorisation(s) | 110 | 115 | 32 | 0 | 0 | 0 | 0 | 0 | 0 |
| Marketing approval(s) | 0 | 0 | 0 | 5 | 0 | 0 | 0 | 0 | 1 |
| Medicinal product(s) | 599 | 329 | 148 | 0 | 0 | 0 | 2 | 0 | 0 |
| Product deviation(s) | 0 | 0 | 0 | 0 | 11 | 0 | 0 | 0 | 0 |
| Regulatory process | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 5 | 0 |
| Quality of . . . | 8 | 1 | 0 | 0 | 1 | 0 | 1 | 4(7[a]) | 0 |
| Quality, safety and efficacy | 7 | 10 | 4 | 0 | 0 | 0 | 2 | 2 | 2 |
| Risk management | 1 | 0 | 7 | 0 | 2 | 0 | 23 | 2 | 2 |

[a] Frequency of term "manufacturing quality."

- Human cell and tissue-based therapeutic goods-related definitions and considerations by the Australian TGA (https://www.tga.gov.au/regulation-basics):

> The Australian Regulatory Guidelines for Biologicals (Aclr1) provide information for manufacturers, sponsors, healthcare professionals and the general public on the legal arrangements in Australia for the supply and use of human cell and tissue-based therapeutic goods. These products are collectively defined as "biologicals." . . . The Biologicals Regulatory Framework is the term for legislation that came into force in 2011 to regulate human cell and tissue-based products as a distinct group of therapeutic goods called "biologicals." The framework is administered by the Therapeutic Goods Administration (TGA), which has produced this document - the Australian Regulatory Guidelines for Biologicals (Aclr1) – to inform manufacturers, sponsors, healthcare professionals and all other interested parties about the framework. . . . The Biologicals Regulatory Framework provides a comprehensive system of assessment and controls that must be completed before products are allowed to be marketed in Australia (premarket), and follow-up and further controls after they are marketed (post-market) . . . or included on the Australian Register of Therapeutic Goods (ARTG) otherwise exempted, approved or authorised.

- ATMPs-related definitions and considerations by the EMA:

> Advanced therapy medicinal products (ATMPs) are medicines for human use that are based on gene therapy, somatic cell therapy or tissue engineering. They offer groundbreaking new opportunities for the treatment of disease and injury. The regulatory framework for ATMPs is established in Regulation (EC) No 1394/2007. (http://www.ema.europa.eu/ema/index.jsp?curl=/pages/home/Home_Page.jsp).
>
> For reasons of clarity, complex therapeutic products require precise legal definitions. Gene therapy medicinal products and somatic cell therapy medicinal products have been defined in Annex 1 to Directive 2001/83/EC, but a legal definition of tissue engineered products remains to be laid down. When products are based on viable cells or tissues, the pharmacological, immunological or metabolic action should be considered as the principle mode of action. It should also be clarified that products which do not meet the definition of a medicinal product, such as products made exclusively of non-viable materials which act primarily by physical means, cannot by definition be advanced therapy medicinal products [10].

Phrase search results are shown in Table 44.6. The phrase "access to" was the one most uniformly present in all documents across the frameworks, although not frequently used altogether (a total of 31 occurrences across all frameworks' documents). The phrases "clinical investigation" and "clinical

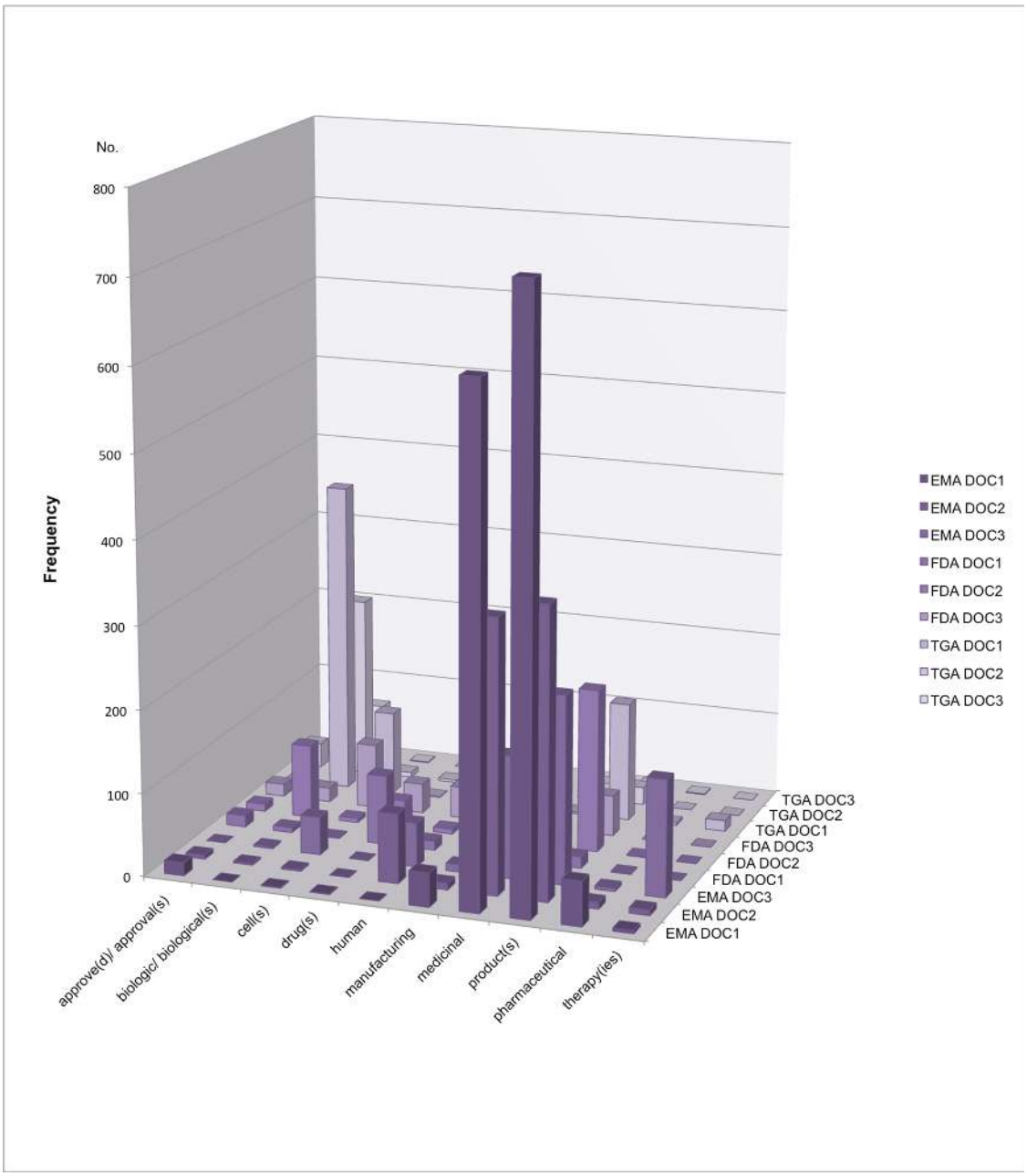



**Figure 44.1** Terminology search graph: frequency of terms in documents (cohort 1).

trial" were interchangeably used by FDA documents, while EMA and TGA documents referred only to "clinical trial." "Human cell(s) and tissue(s)" and "human cells" were only referred to by a single document in each framework (9 occasions in EMA DOC3, 12 in FDA DOC3, and 12 in TGA DOC1), while "investigational new drug" was mentioned only in two FDA documents (a total of 32 occasions in FDA DOC1 and DOC2).

The TGA documents referred often to "manufacturing" (along with the other two frameworks' documents to a lesser extent) and "approved/unapproved," while mentioning "cell(s)" over 100 times in only one of the TGA documents (TGA DOC1). The TGA framework relied heavily on the terms "product(s)" and "risk management," on 171 and 27 occasions respectively. "Risk management" was mentioned less frequently in the EMA documents and only twice in all FDA documents. The term "therapy/ies" was not mentioned in any of the FDA documents and was seldom used in the TGA documents (13 times in total), while it was mentioned 141 times in the EMA DOC3.

Overall, the EMA framework's documents referred to "medicinal products" and "marketing authorisation(s)," the FDA documents discussed "drug(s)" or "biologic(s)," and the TGA

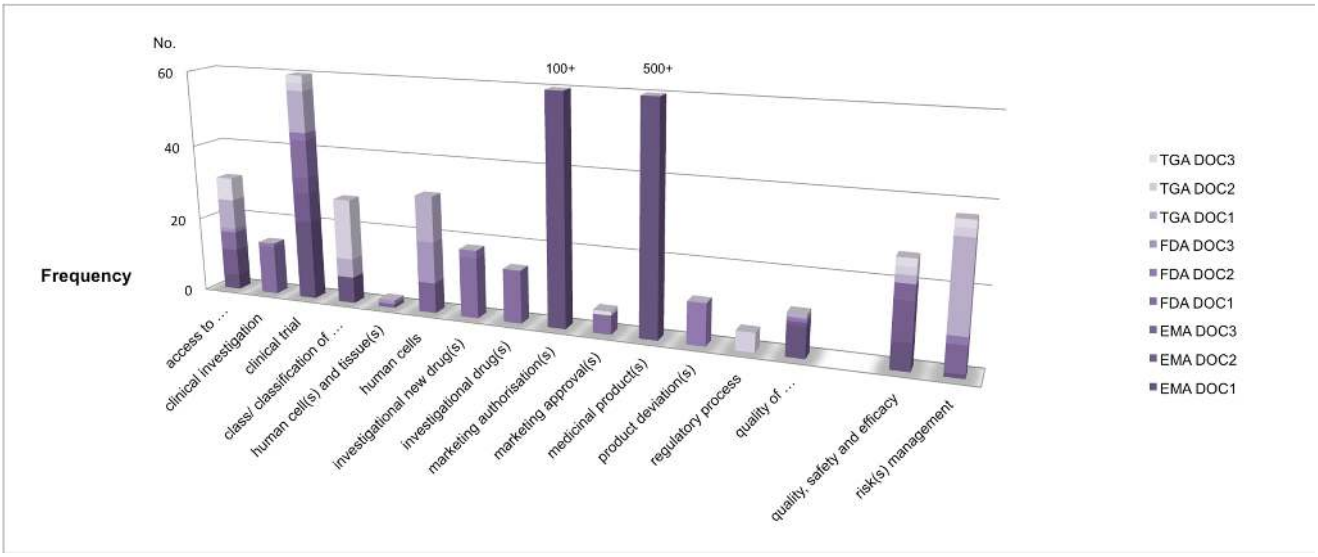



**Figure 44.2** Terminology search graph: frequency of phrases in documents (cohort 1).

documents mentioned "biological(s)." Hence, all the documents signified "product" as such – although referred to it using different terminology ("medicinal product" versus "drug/biologic" versus "biological"). Furthermore, the EMA documents were mostly concerned with "marketing authorisation(s)" while mentioning "quality, safety and efficacy." In the same manner, the FDA documents discussed "drug(s)" or "investigational drug (s)" that were not cited in the other two frameworks at all. They also utilized "product deviation" exclusively and used "clinical investigation" and "clinical trial" interchangeably.

The results reported in this chapter reveal numerous differences between the regulatory frameworks' high-tier documents and the terminology they employ. For instance, "marketing authorisation" was exclusively utilized by the EMA regulatory framework, as well as the phrase "medicinal products." Neither of these was mentioned at all in the FDA and the TGA frameworks' documents. "Class" or "classification of" were not mentioned at all in the FDA documents, while the same phrases were utilized minimally in the EMA documents (seven occurrences) but more frequently in the TGA documents (54 occurrences).

"Quality, safety and efficacy" was a phrase barely used in the EMA and the TGA documents,and it was not present in any of the FDA documents. This also applied to the phrases "quality of" or "manufacturing quality."

## 44.6 Regulatory science

Biologic drugs must be developed and manufactured through disciplined and specific mechanisms [3]. This is the case even when full compliance with traditional concepts of pharmaceutical manufacturing practice is sometimes not possible (although strived for), particularly for life-saving therapies and new modalities in investigational drug development [12]. These systems incorporate considerations of risk/benefit ratios and include mechanisms for transparent and accountable release of products [16].

Conditions of product requirements and release are established and outlined in a spectrum of documents from the high-tier regulatory documents down to the procedural documents. Hence, the documents outlining the highest level of regulatory requirements in three mature regulatory frameworks were analyzed by a documentary analysis method in order to establish concomitance between different regional regulations applicable to biologic drugs as a source of a specialized knowledge in regulatory science.

The Commissioner of the FDA stated "that a failure to invest 'adequately' in regulatory science has been one of the reasons why fewer new drugs have been making it to the market" [30]. In October 2010 the FDA promised to bring its "regulatory science" into the 21st century [31], and in August 2011 it issued its strategic plan for regulatory science [32] called Advancing Regulatory Science at FDA [18]. The Common Fund's Regulatory Science program was initiated in the second half of 2010 as a partnership between the US National Institutes of Health and the FDA [33]. At the same time, the EMA stated on its website that "the European Medicines Agency is a key leader in the development and application of regulatory science" [34] and referred to the conference hosted by the agency in December 2010, which resulted in the report "Regulatory science: are regulators leaders or followers?" [35].

The origin of the term regulatory science is unknown, but it is believed that it was created in the 1970s when the newly formed US Environmental Protection Agency was required to make decisions based on incomplete and sometimes nonexistent scientific information [36]. Although there is still some debate as to

whether it is necessary to use this new terminology "as 'science is science' regardless how it is applied" [36], the FDA currently defines regulatory science as "the science of developing new tools, standards, and approaches to assess safety, efficacy, quality, and performance of all (FDA) regulated products" [37]. There is an undeniable need to capture regulatory knowledge and expertise required for the development of future regulatory policies to enable safe application of new biologic drugs [38].

## 44.7 Interpretation of the analysis of the documents

This chapter has described the processes that were used to develop a method of manual documentary analysis for high-tier regulatory documents. Although the data were studied out of context and the selectiveness of the data or representativeness of the sample can be questioned, this approach to documentary analysis is particularly useful when the researcher is faced with a vast number of complex documents with no common format, scope, or terminology.

Similar studies aimed at analyzing or comparing regulatory documents have been performed in the pharmaceutical industry, and other industries such as aviation and education [39–42]. Irrespective of the area and a specific methodology (manual or automated documentary analysis) employed in each study, they have added benefit to their respective areas. The purpose of these studies was either to provide a procedure for assessing connections between the current regulations and incorporation of new requirements [39], to increase understanding and knowledge about the trends in success of regulatory submissions [40], or to compare the regulatory versus the educational value of documents – for example, guidances for undergraduate education for medical practitioners [41]. Some of the research looked into establishing a clear definition of requirements or identifying inconsistencies between those definitions [42].

In the process of choosing the documents for the study, an ontological approach was adopted that assumed written words, texts, and documents are meaningful constituents of the process in the way in which they are changed and utilized [17]. In addition, an epistemological position was adopted that suggested that texts, documents, and written material can provide or count toward evidence of these ontological properties. The benefits of studies aimed at analyzing or comparing regulatory documents have been already recognized in the health care [39], the pharmaceutical industry [40,42], and the aviation and education [41] fields, while qualitative documentary review and analysis has demonstrated benefits to a number of other sectors, such as political science [43], environmental health research [44], ethics [45], and the World Health Organization's health economics and policy-making research [46].

It was observed that the main tasks in documentary analysis are a thorough understanding of documents analyzed, familiarity with relevant terminological differences, and robust quality assurance of the methodology [47–49]. The main advantages for the use of existing documents in a research study are that the data are usually easy to access, it takes a relatively short period of time to collect the data, and to some extent it provides a relatively inexpensive form of data collection [27]. In addition, the documents are unbiased by the data collection process (i.e., nonreactive) and may be useful for hypothesis and problem formulation. Nonreactivity is one of the methodological strengths of documentary analysis – "the data selection method itself generally does not change the data being collected" [28]. The stability of a measure refers to the extent to which the same results are obtained on repeated administrations of the instrument and it focuses on the instrument's susceptibility to "extraneous factors from one administration to the next" [49]. It was considered appropriate to rely on the "stability" of the documents as a retest measurement facility due to the nature of high-tier documents available to the public and researchers. Reliability refers to the accuracy of the research methods and techniques [50,51]. The process of reading, understanding, selecting, and comparing documents added a further dimension of construction as well as reflexivity [52]:

> textual analysis involves the mediation between the frames of reference of the researcher and those who produced the text . . . the researcher's own frame of reference becomes the springboard from which the circle is entered, and so the circles reaches back to encompass the dialogue between the researcher and the text.

Careful consideration of the validity of data generation requires a precise application of the logic used previously in determining methodological approaches – the chosen methodology has to be more valid than other methodological approaches [19]. To some extent there is a blurring of the distinction between validity and reliability. The main question posed here was how well the documents analyzed represented the concepts of the research or the hypothesis in an accurate, authentic, and relevant manner [29]. Since the issue of similarities and differences between the regulatory approaches was examined, it appears appropriate to use high-tier regulatory documents that have established the hallmark of these frameworks. It would be difficult to justify use of any other documentary sources for this type of research since the other documents, such as lower tier procedural texts or guidelines, are either overly specialized in their aim or scope or not considered as mandatory for every product.

Generally, qualitative research cannot entirely rely on standardized procedures to deal with concepts such as bias and reproducibility [50]. In this process, reproducibility is often replaced with transparency or an audit trail (a record of the researcher's design decision) about gaining access, collecting, and analyzing the data or about the actual research design [51]. Manual analysis with its quantified data (i.e., frequencies of terms and phrases) can be repeated accurately on many occasions and with no operator dependency. The only variable potentially influencing the reproducibility of the study relies upon selection

of the documents to be analyzed. Construct validity concerns the degree to which results of documentary analysis of documents reflects the underlying theoretical concepts or exposure pointers. This would be "the extent to which the theoretical concepts have been successfully operationalised" and "the process of translating research concepts into measurable phenomena" [27]. Validity of the construct was established by the assumption that differences in the regulatory frameworks were dictated by different historical and cultural backgrounds, regional dynamics, and various complexities of the biologic drug development. Nevertheless, some shared concerns were considered an underpinning factor for similarities among the regulatory frameworks, such as public and individual safety and a uniform scientific knowledge. Validity of interpretation is another way to think about the validity of the research. It is concerned with the data analysis validity and the interpretation on which it is based; it is contingent upon the end result, including how that interpretation was reached [19]. The validity of interpretation was established in two ways. First, by making the process of reaching interpretations as transparent as possible. Second, by following the same logic in making methodological choices and identifying the sources to be used – the documents analyzed.

Furthermore, the intent of the research was to provide some level of generalization, since it examined a broad set of regulations in three areas of the developed world: Europe, the USA, and Australia. Generalization is sometimes perceived in two distinct ways: empirical (i.e., one empirical population is representative of a wider population) and theoretical [19]. Theoretical generalization is often seen as more productive since it encompasses a range of strategies based on different logics, some of which are more "theoretical" than others (i.e., attaining a wider perspective) [19]. The aim of the theoretical generalization of this research was to demonstrate that high-tier regulatory documents are a central representation of the regulatory frameworks from which they are drawn. The comparison aimed to contribute more to the generalization than a simple statement of similarity or discrepancy.

It was also designed to pose questions for future regulatory studies. What questions do all regulatory agencies want answered? What are the specific areas that they wish to emphasize? In what specific areas do they converge or diverge and how can the lessons learned from a conventional pharmaceutical model be applied to biopharmaceutical development? It is well known that the biggest risk in drug development is failure to convince a regulator that a given therapeutic product meets the requirements for the next stage of development or marketing approval. It is also becoming clear that regulatory education and precedence information analysis, along with thorough knowledge of regulatory framework(s) and their focus, are the most acceptable development strategies for a smarter product development pathway. Not only does it require fewer clinical and nonclinical trials while reducing time and related cost [53], but it also potentially brings development of a biologic drug closer to market approval stage in different regions [54,55].

## 44.8 Conclusions

This chapter draws attention to the importance of understanding the complexity of a global regulatory environment applicable to biologic drugs, including stem cells and their progeny. It demonstrates considerable differences in the regulatory approach to biologic drugs' development and application, particularly with respect to terminology and definitions used in different regional regulatory frameworks. The EMA documents were mostly concerned with "marketing authorisation(s)," while occasionally mentioning "quality, safety and efficacy"; the FDA documents discussed "drug(s)" or "investigational drug(s)" that were not cited in the other two frameworks at all, utilized "product deviation" exclusively, and used "clinical investigation" and "clinical trial" interchangeably. While the most frequent words used in the EMA documents were "product(s)" along with "medicinal," the term "drug(s)" was consistently mentioned within all FDA documents but seldom mentioned in the other two frameworks. The TGA documents featured "biologic/biological(s)" most consistently, while "cell(s)" was most utilized in a document from the FDA framework and the TGA framework.

Regardless of these disparities between the three frameworks, this study identifies some conjoint aspects: the focus on the "product," whether it is referred to as "medicinal product," "drug/biologic" or "biological" is an example. Similarly, the terms "drug(s)," "cell(s)," and "biologic/biological(s)" were to some extent used across the entire spectrum of documents analyzed. All documents use the term "human" in a comparable manner.

Although the data were studied out of context and the selectiveness of the data or representativeness of the samples can be questioned, this approach to documentary analysis is particularly useful when the researcher is faced with a huge number of complex documents with no common format, scope, and terminology.

## References


1 Burger SR. Current regulatory issues in cell and tissue therapy. *Cytotherapy* 2003;**5**:289–298.

2 Dutton RJ, Scharer JM. *Advanced Technologies in Biopharmaceutical Processing*. Ames, IA: Blackwell, 2007.

3 Prince HM, Wall DP, Stokes HK, *et al.* Cell processing for clinical trials and commercial manufacture. *Cell Gene Ther* 2004;**5**:15–21.

4 Kaiser J. *US Federal Court Says Stem Cell Treatments Are Drugs.* http://www.sciencemag.org/news/2012/07/us-federal-court-says-stem-cell-treatments-are-drugs (accessed April 10, 2016).

5 Freeman M, Fuerst M. Does the FDA have regulatory authority over adult autologous stem cell therapies? 21 CFR 1271 and the emperor's new clothes. *J Transl Med* 2012;**10**:60–65.

6 Rosenblast B. Meeting regulatory challenges for cell-based therapies (Supplement). *BioProcess Int* 2012;**10**(3):8–11.

7 Australian Government, Department of Health and Ageing, Therapeutic Goods Administration. *Resource guide: Overview of the Biologicals Framework, November* 2010. http://web.archive.org/

web/20110501000000*/http://www.tga.gov.au/pdf/consult/consult-biologicals-tgregs-1011-guide.pdf (accessed April 18, 2016).
8 US FDA. *Drugs@FDA Glossary of Terms*. http://www.fda.gov/Drugs/InformationOnDrugs/ucm079436.htm (accessed April 10, 2016).
9 Rader R. (Re)defining biopharmaceutical. *Nat Biotechnol* 2008;**26**(7):743–751.
10 Regulation (EC) No 1394/2007 of The European Parliament and of the Council, Official Journal of the European Union (10.12.2007). Available at http://ec.europa.eu/health/files/eudralex/vol-1/reg_2007_1394/reg_2007_1394_en.pdf (accessed April 10, 2016).
11 To thwart disease, apply now (Editorial). *Nature* 2008;**453**(7197):823.
12 Kirouac DC, Zandstra PW. The systematic production of cells for cell therapies. *Cell Stem Cell* 2008;**3**:369–381.
13 EMA. *Final Report on the International API Inspection Pilot Programme,* 16 June 2011, EMA/453741/2011. http://www.ema.europa.eu/docs/en_GB/document_library/Report/2011/07/WC500108655.pdf (accessed April 10, 2016).
14 Halme DG, Kessler DA. FDA regulation of stem-cell-based therapies. *N Engl J Med* 2006;**355**:1730–1735.
15 *FDA Guidance for industry: PAT – A framework for innovative pharmaceutical development, manufacturing, and quality assurance*. US FDA, 2004. http://www.fda.gov/downloads/Drugs/ . . . /Guidances/ucm070305.pdf (accessed April 18, 2016).
16 Burger SR. CTP/GMP cell engineering for cell and gene therapies. *Bioprocess J* 2003;**2**:66–69.
17 European Commission, Enterprise and Industry. *The Financing of Biopharmaceutical Product Development in Europe,* Final Report, October 2009. http://ec.europa.eu/DocsRoom/documents/1416/attachments/1/translations/en/renditions/pdf (accessed March 20, 2016).
18 US Department of Health and Human Services, Food and Drug Administration. *Advancing Regulatory Science at FDA, Strategic Plan: August* 2011. http://www.fda.gov/downloads/ScienceResearch/SpecialTopics/RegulatoryScience/UCM268225.pdf (accessed April 10, 2016).
19 Mason J. *Qualitative Researching*, 2nd edn. Sage Publications: London, 2002.
20 EMA. *Implementing the European Medicines Agency's Road Map to 2015: The Agency's Contribution to Science, Medicine, Health – From Vision to Reality*, 6 October 2011, EMA/MB/550544/2011. http://www.ema.europa.eu/docs/en_GB/document_library/Other/2011/10/WC500115960.pdf (accessed April 10, 2016).
21 Ilic N, Khalil D, Hancock S, *et al.* Regulatory considerations applicable to manufacturing of human placenta-derived mesenchymal stromal cells (MSC) used in clinical trials in Australia and comparison to USA and European regulatory frameworks. In: LG Chase, MC Vemuri (eds), *Mesenchymal Stem Cell Therapy*. Stem Cell Biology and Regenerative Medicine Series. Springer/Humana Press: Heidelberg, 2013; pp. 373–404.
22 US FDA. *Development & Approval Process (CBER)*, 2010. http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/default.htm (accessed April 10, 2016).
23 US FDA, CDER, CEBR, ICH. *Manual of Policies & Procedures (CDER)*, 2016. http://www.fda.gov/AboutFDA/CentersOffices/OfficeofMedicalProductsandTobacco/CDER/ManualofPoliciesProcedures/default.htm (accessed April 10, 2016).
24 US FDA. *New Drug Application (NDA) Process (CBER)*, 2010. http://www.fda.gov/BiologicsBloodVaccines/DevelopmentApprovalProcess/NewDrugApplicationNDAProcess/default.htm (accessed April 10, 2016).
25 Anderson M. *FDA abandons Declaration of Helsinki for international clinical trials*. Social Medicine Portal, 1 June 2008. http://www.socialmedicine.org/2008/06/01/ethics (accessed April 18, 2016).
26 The International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH). *Open Consultation*. http://www.ich.org/products/open-consultation.html (accessed April 18, 2016).
27 Thomas J, Harden A. Methods for the thematic synthesis of qualitative research in systematic reviews. *BMC Med Res Methodol* 2008;**8**:45–55.
28 Bailey KD. *Methods of Social Research*, 2nd edn. Free Press: New York, 1982.
29 Robson C. *Real World Research*, 2nd edn. Blackwell: Oxford, 2002.
30 Sukkar E. Lack of investment in regulatory science is part of "innovation gap," believes FDA commissioner. *BMJ* 2011;**343**:d8017.
31 Lowes RR. *FDA approval of 'novel' new drugs up sharply in* 2014. http://www.medscape.com/viewarticle/838536 (accessed April 10, 2016).
32 US FDA. *Strategic Plan for Regulatory Science: Advancing Regulatory Science at FDA: A Strategic Plan (August* 2011 *)*. http://www.fda.gov/ScienceResearch/SpecialTopics/RegulatoryScience/ucm267719.htm (accessed April 18, 2016).
33 NIH. *Regulatory Science: Overview*, 2015. www.commonfund.nih.gov/regulatoryscience/overview (accessed March 20, 2016).
34 European Medicines Agency. *Medicines and emerging science*. http://www.ema.europa.eu/ema/index.jsp?curl=pages/special_topics/general/general_content_000339.jsp&mid=WC0b01ac05800baed8 (accessed April 18, 2016).
35 EMA Report: Regulatory science: are regulators leaders or followers? http://www.ema.europa.eu/docs/en_GB/document_library/Agenda/2011/01/WC500100967.pdf (accessed April 18, 2016).
36 The Institute for Regulatory Science. *What is Regulatory Science?* 2015. http://www.nars.org/2-uncategorised/15-what-is-regulatory-science (accessed April 18, 2016).
37 US FDA. *Advancing Regulatory Science: Moving Regulatory Science into the 21st Century*. 2016. http://www.fda.gov/ScienceResearch/SpecialTopics/RegulatoryScience/default.htm?utm_campaign=Goo (accessed April 18, 2016).
38 Johns Hopkins Bloomberg School of Public Health, Centre of Excellence in Regulatory Science and Innovation (CERSI). *Research*. http://www.jhsph.edu/research/centers-and-institutes/center-of-excellence-in-regulatory-science-and-innovation/research/ (accessed April 18, 2016).
39 Munns TE, Beard RE, Culp AM, Murphy DA, Kent RM. *Analysis of Regulatory Guidance for Health Monitoring,* NASA/CR-2000-210643, December 2000. http://ntrs.nasa.gov/archive/nasa/casi.ntrs.nasa.gov/20010017836_2001019668.pdf (accessed April 10, 2016).
40 Putzeist M, Mantel-Teeuwisse AK, Gispen-De Wied CC, Hoes AW, Leufkens HG. Regulatory Scientific advice in drug development: does company size make a difference? *Eur J Clin Pharmacol* 2011;**67**:157–164.

41 Gray T, Grant J. Good medical practice or CanMEDS for education? *J Clin Pathol* 2012;**65**(6):565–567.
42 Rozet E, Ceccato A, Hubert C, *et al.* Analysis of recent pharmaceutical regulatory documents on analytical method validation. *J Chromatogr* 2007;**1158**:111–125.
43 Wesley JJ. Qualitative document analysis in political science. In *T2PP Workshop*, 9–10 April 2010, Vrije Universiteit, Amsterdam.
44 Kangsen Scammell M. Qualitative environmental health research: an analysis of literature, 1991–2008. *Environ Health Perspect* 2010;**118**:1146–1154.
45 Sixsmith J, Murray CD. Ethical issues in the documentary data analysis of Internet posts and archives. *Qual Health Res* 2001;**11**: 423–432.
46 Hanney SR, Gonzalez-Block MA, Buxton MJ, Kogan M. The utilisation of health research in policy-making: concepts, examples, and methods of assessment. *Health Res Policy Syst* 2003;**1**:2.
47 Miles MB, Huberman AM. *Qualitative Data Analysis*, 2nd edn. Sage Publications: Thousand Oaks, CA, 1994.
48 Patton MQ. *Qualitative Research & Evaluation Methods*, 3rd edn. Sage Publications: Thousand Oaks, CA, 2002.
49 Weber RP. *Basic Content Analysis*, 2nd edn. Sage Publications: Thousand Oaks, CA, 1990.
50 Creswell JW. *Qualitative Inquiry & Research*, 2nd edn. Sage Publications: Thousand Oaks, CA, 2007.
51 Maxwell JA. *Qualitative Research Design*, 2nd edn. Sage Publications: Thousand Oaks, CA, 2005.
52 Scott J. *A Matter of Record*. Polity Publishing, 1990.
53 Kumar M. *Taming the Regulatory Beast: Role of Regulatory Trend Analysis in Successful FDA Approval* (Editorial). http://www.mastercontrol.com/newsletter/pharmaceutical/regulatory_trend_analysis_fda_approval_1009.html (accessed April 10, 2016).
54 EMA. *European Medicines Agency sees Benefits of Interaction with Japanese Regulators,* 30 July 2012. http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2012/07/news_detail_001564.jsp&mid=WC0b01ac058004d5c1 (accessed April 10, 2016).
55 Human Tissue Authority (UK), Advanced Therapy Medicinal Products FAQs. https://www.hta.gov.uk/policies/regenerative-medicine-and-regulation-advanced-therapies-medicinal-products-atmps (accessed April 18, 2016).

SECTION VI

# The therapeutic application of mesenchymal stromal cells

CHAPTER 45

# The use of mesenchymal stromal cells in acute and chronic heart disease

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## 45.1 Introduction

Despite the recent decline in deaths attributable to cardiovascular disease (CVD), it remains the leading cause of death not only in the USA but alsothe entire developed world [1]. More Americans die each year from CVD than cancer and chronic lower respiratory diseases combined, and the economic burden in medical costs alone is projected to reach $918 billion by the year 2030 [1]. Therefore, innovative, cost-effective treatments are needed. The heart is one of the least regenerative organs, and the standard of care for the treatment of CVD has been restricted to either mitigating further damage or heart transplantation. However, in the past several years there has been an increased focus on overcoming this lack of regenerative potential by the therapeutic use of stem/progenitor cells. Mesenchymal stromal/stem cells (MSCs) are a particularly attractive candidate due to their ease of isolation [2], immune privilege [3], ability to stimulate endogenous cardiac stem cells (CSCs) [4], and the release of beneficial paracrine factors [5]. In this chapter we will examine the therapeutic use of MSCs for acute and chronic ischemic heart disease in the preclinical and clinical setting as well as the possible mechanisms involved in their reversal of cardiac remodeling.

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## 45.2 The biology of acute and chronic ischemic cardiomyopathy

Occlusion of one or more major coronary arteries, even if temporary, produces myocardial ischemia, and the resulting lack of oxygen and subsequent depletion of creatine phosphate inevitably leads to a shift from aerobic metabolism to anaerobic glycolysis in order to meet the energy demands of the heart [6]. This shift in the energy-generating pathway is characterized by a severely hypoxic environment leading to acidosis due to the accumulation of glycolytic intermediates, primarily lactate [6]. As the energy demand of the cardiomyocytes exceeds supply, adenine diphosphate increases and

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adenylate kinase is activated, leading to a buildup of adenosine monophosphate (AMP) [7]. As AMP accumulates it is progressively degraded into nucleosides and bases, which eventually efflux from cardiomyocytes. The progressive catabolism of ATP, glycogen stores, and more catabolites during cardiomyocyte metabolism leads to an increased osmolar load, and therefore cell swelling [6]. As a consequence of this increased load, $Na^+/K^+$-ATPase, $Na^+/H^+$, and $Na^+/Ca^{2+}$ ion gradients are disrupted, leading to the influx of calcium. When intracellular calcium reaches a threshold level, the activation of specific phospholipases and proteases ensues, leading to autolysis of the cytoskeletal attachment complexes, subsequent breaks in the plasmalemma (cell membrane), and myocyte leakage [6]. If ischemia continues for 40–60 min without reperfusion, cardiomyocytes begin the transition from reversible to irreversible injury [7], beginning in the subendocardial layer and proceeding to the subepicardial layer [8]. While the precise transition point from reversible to irreversible injury remains unknown, it is the disruption of the sarcolemma, the fine transparent tubular sheath that envelops the fibers of striated muscles, that indicates definitive myocardial death [8]. Therefore, the lack of oxygen to myocardial tissue following a myocardial infarction (MI) leads to hypoxia followed by acidosis, which contributes to massive cell death of all cardiac cell types, including cardiomyocytes, vascular smooth muscle cells, fibroblasts, and endothelial cells. A collagenous avascular scar replaces necrotic tissue, causing adverse cardiac remodeling and eventually heart failure.

The structural and functional effects of the aforementioned myocardial ischemia were originally considered permanent, as the heart was thought to be a terminally differentiated postmitotic organ [9]. However, evidence of native myocyte proliferation [10] and the discovery of endogenous CSCs [11] highlighted the potential for reversal of CVD damage by optimizing the heart's regenerative potential. Exogenous stem cell therapy seeks to reverse cardiac remodeling by engraftment into the myocardium and differentiation as well as promoting resident cardiomyocyte cell cycling and endogenous CSC proliferation and differentiation. While early *in vitro* and preclinical studies focused on pluripotent stem cells, including embryonic stem cells and induced pluripotent stem cells because of their multipotential differentiation capacity, the ethical concerns of the former and teratoma formation by both, shifted the focus toward other viable cell options, including unfractionated bone-marrow-derived mononuclear cells (BM-MNCs) [12,13]. While early clinical trials utilizing BM-MNCs provided promising results [14,15], more recent clinical trials have failed to document the regenerative capability of these cells [16–19]. These conflicting results have shifted the focus again toward other bone marrow stem cell populations, with one of the most promising being MSCs. In addition to the ability of MSCs to engraft [20] and to contribute to reverse cardiac remodeling [4,20,21], MSCs provide the possibility for use as an allogeneic off-the-shelf product because of their immunosuppressive properties [22–24].

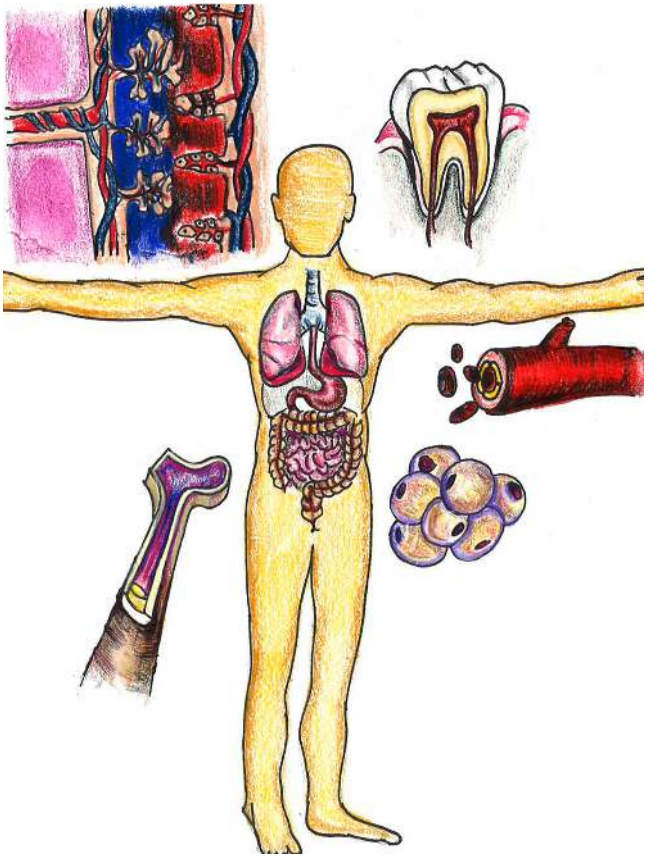


**Figure 45.1** MSCs reside in nearly every tissue of the body, including, but not limited to, bone marrow, adipose, lung, gut, amniotic fluid, placenta, umbilical cord, Wharton's jelly, dental pulp, and also in the circulation. (See color plate section.)

## 45.3 Characterization of mesenchymal stromal/stem cells

MSCs were originally isolated from the bone marrow [25] but have recently been shown to reside in virtually every tissue, including fat, lung, gut, amniotic fluid, placenta, umbilical cord, Wharton's jelly, dental pulp, and also in the circulation [26,27] (Figure 45.1).

In 1970, Friedenstein *et al.* discovered a population of adult stem cells that comprised 0.001% of the bone marrow of guinea-pigs [25]. These cells exhibit the necessary characteristics of stem cells, in that they have the capacity for self-renewal and multilineage differentiation and also exhibited plastic adherence *ex vivo* [28,29]. Their ease of isolation and therapeutic potential led to a swift transition from early preclinical studies into clinical trials in a variety of diseases. In 2006 the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy established the minimal criteria for MSC characterization in order to provide consistency and clarification as the cell field progressed. These criteria included (1) adherence to plastic when maintained in standard culture conditions; (2) expression of surface markers CD105, CD73, and CD90 while

lacking expression of CD45, CD34, CD14, CD11b, CD79α, CD19, and human leukocyte antigen (HLA)-DR; and (3) the ability to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* [30]. However, it is important to note that these standards are the minimal defining characteristics. MSCs may differentiate into a variety of both mesoderm- [31–33] and non-mesoderm-derived tissues [34], although the latter is controversial, particularly with regard to efficacy. MSCs have become one of the most prominent contenders as a therapeutic treatment for acute and chronic heart disease for a variety of reasons, perhaps the most distinct being their capacity for immunomodulation.

### 45.3.1 Immunomodulatory properties

MSCs are particularly effective in preventing abnormal cardiac remodeling in the setting of acute MI (AMI) owing to their potent immunomodulatory properties (Figure 45.2), which are particularly important for the use of allogeneic MSCs.

The basis of MSC immune privilege is their low to moderate expression of HLA and major histocompatibility complex (MHC) class I and their lack of MHC class II molecules, which are involved in mediating interactions with leukocytes [35]. They also lack Fas ligand and co-stimulatory molecules, including CD80 and CD86 (B7.1 and B7.2 respectively) and CD40 ligand [36]. These properties have enabled the therapeutic use of MSCs as a convenient off-the-shelf ready-to-go therapeutic product in a number of conditions, including treatment-refractory graft-versus-host disease and acute and chronic ischemic cardiomyopathy. Their ability to hide from the immune system enables MSCs to survive/differentiate as both an allograft and a xenograft [36,37].

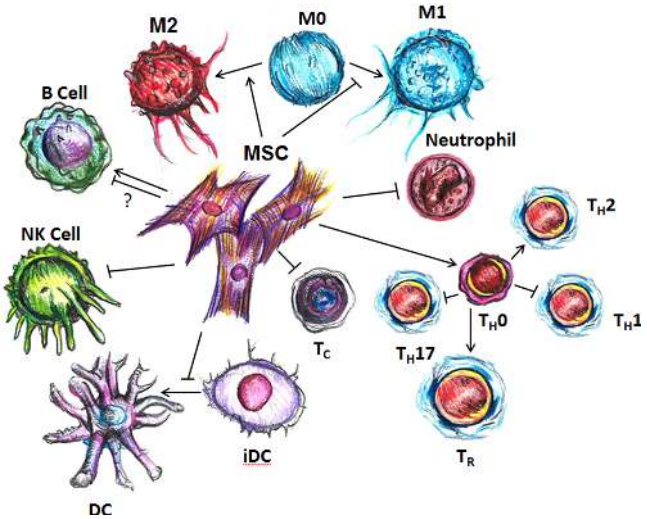



**Figure 45.2** The immunomodulatory properties of MSCs. An overview of the relationship between MSCs and the immune system. MSCs suppress natural killer (NK) cell activity through the secretion of soluble factors and downregulation of their activating receptors. The maturation of dendritic cells (DCs) is inhibited through the secretion of interleukin (IL)-6, which indirectly inhibits T cell activation. T cell activity is regulated by MSCs via multiple pathways. While MSCs inhibit the proliferation of both cytotoxic T ($T_C$) and helper T ($T_H$) cells by inducing cell cycle arrest, they promote differentiation of regulatory T ($T_R$) cells and the anti-inflammatory $T_H2$ cells. Additionally, MSCs preferentially promote monocyte differentiation into the anti-inflammatory M2 macrophage phenotype while inhibiting M1 macrophage differentiation. MSCs delay neutrophil apoptosis through the release of IL-6 while simultaneously decreasing the production of reactive oxygen species. The effect of MSCs on the humoral adaptive immune system remains unclear, with some studies indicating that MSCs inhibit B cell proliferation and subsequent antibody production, while others have found that MSCs elicit the opposite effects. (See color plate section.)

MSCs exert their immunomodulatory effects by interacting with both the innate immune system (macrophages, neutrophils, NK cells, and DCs) and adaptive immune system (T cells and B cells). After AMI, macrophage colony-stimulating factor promotes the maturation of monocytes to macrophages [38]. M1 macrophages, activated in the presence of nitric oxide (NO), secrete proinflammatory cytokines and are involved in debris cleaning in the initial stages of an AMI [39]. MSCs promote monocyte differentiation into the alternatively activated anti-inflammatory M2 phenotype involved in cardiac repair and angiogenesis, while still retaining the debris cleaning properties of the M1 phenotype [39]. Neutrophils are activated in response to tissue damage following MI, and produce reactive oxygen species to kill microorganisms [40]. MSCs secrete IL-6, which decreases the production of reactive oxygen species (thus decreasing the respiratory burst) and delays neutrophil apoptosis, therefore preventing further tissue damage while maintaining microbial phagocytic activity [40]. (The respiratory burst is the rapid release of reactive oxygen species, such as superoxide radical and hydrogen peroxide.) After an AMI, DCs activate the adaptive immune system through presentation of antigens to T cells. MSCs not only inhibit the maturation of monocytes to DCs but also indirectly inhibit T cell activation, breaking the link between the innate and adaptive immune systems [41]. NK cells are cytotoxic leukocytes that respond to a viral infection or stressed cells, such as those affected by an AMI. MSCs suppress NK cell activity by secretion of soluble factors and by downregulating their activating receptors, which in turn leads to further NK suppression [3].

T cells involved in the adaptive immune system consist of $T_H$ cells, $T_R$ cells, and $T_C$ cells. While $T_H$ and $T_C$ cells are involved in the apoptosis of antigen-presenting cells, regulatory T cells mediate the immune response. MSCs suppress the proliferation of $T_H$ and $T_C$ cells by inducing cell cycle arrest in the $G_0/G_1$ phase through various pathways. They also exert their effects through lineage commitment by inhibiting proinflammatory $T_H1$ and $T_H17$ differentiation [42], while promoting the differentiation of anti-inflammatory $T_H2$ cells [43]. Finally, MSCs also induce $T_R$ cell differentiation, which creates a negative feedback loop to inhibit T cell maturation [42,44]. Therefore, MSCs suppress the adaptive immune system both directly and indirectly.

B cells are also involved in the adaptive immune system and are responsible for antibody production, which leads to macrophage and neutrophil phagocytosis of antigen-presenting cells. In an AMI, inflammation leads to antibody production,

macrophage recruitment, and subsequent tissue damage. Similar to T cells, MSCs are capable of arresting B cells in the $G_0/G_1$ phase, thereby halting proliferation and continued myocardial destruction [45]. However, while some researchers have demonstrated that MSCs inhibit B cell-mediated antibody production [45], others have shown the opposite [46]. Therefore, the effect of MSCs on the humoral adaptive immune system requires further investigation.

Thus, MSCs can play a significant immunomodulatory role after MI through cell–cell interaction and the secretion of multiple soluble factors (the paracrine effect) and are potent cardio-protective agents. They also secrete a variety of cytokines and growth factors involved in scar reduction and activation of matrix metalloproteinases (MMPs) involved in degradation of the extracellular matrix. Therefore, MSCs play an important role in both the prevention and reduction of fibrosis.

### 45.3.2 Antifibrotic effects

One of the most significant factors leading to adverse cardiac remodeling following an AMI is fibrosis [47]. As stated earlier, after an AMI, if the tissue is not quickly reperfused, irreversible cell death occurs, leading to the replacement of necrotic myocardium with scar tissue. This process entails the destruction of the myocardial extracellular matrix followed by the formation of a provisional matrix composed of fibronectin, laminin, collagen, and other proteins and finally the maturation of a collagen-based scar [48]. While the antifibrotic effect of MSCs in both the preclinical and clinical settings has been well described, the exact mechanism is poorly understood. *In vitro* studies have shown that MSCs decrease the viability of myofibroblasts and subsequently increase their ability to degrade the extracellular matrix [49]. Decreased fibroblast viability was assessed by reduced expression of α-smooth muscle actin, a marker of myofibroblast differentiation. MSC-conditioned medium stimulates the activity of MMP2/MMP9 in cardiac fibroblasts, while decreasing the expression of tissue inhibitor metalloproteinases (TIMPs), namely TIMP-2. This expression demonstrates a shift in ECM remodeling from production to degradation, which therefore inhibits the subsequent deposition of collagen. Mias *et al.* also demonstrated that MSC-treated mice presented with decreased type I and type III collagen deposits at 2 months after MI [49]. MSCs also secrete specific growth factors and cytokines involved in the reduction of scar tissue, including hepatocyte growth factor (HGF) and heme oxygenase, both possible key factors involved in MMP and TIMP regulation [49–51]. Cumulatively, these studies in addition to the results of preclinical and clinical studies have led us closer to understanding the mechanisms involved in MSC-induced scar reduction.

### 45.3.3 Cardiomyogenesis *in vitro* and *in vivo*

The antifibrotic effects of MSCs are accompanied by both cardiomyogenesis and neovascularization. MSCs promote cardiomyogenesis by engrafting into the myocardium and differentiating into cardiomyocytes as well as by stimulating endogenous CSC differentiation and promoting resident cardiomyocyte cell turnover. The ability of MSCs to differentiate into cardiomyocytes *in vitro* [52] inspired subsequent small and large animal studies [4,20,53]. *In vitro* studies demonstrated the capacity of cardiomyocytes to adopt a cardiac phenotype by co-culture with 5-azacytidine, other cardiac myocytes, or pro-cardiogenic factors, including bone morphogenetic protein (BMP)-2, HGF, transforming growth factor beta (TGF-β), and Jagged1 [5,54–57]. Xu *et al.* co-cultured MSCs with 5-azacytidine and demonstrated differentiation into cardiomyocytes as noted by the expression of known myogenic markers, including α-cardiac actin, β-myosin heavy chain, and desmin, as well as the appearance of cytoplasmic myofilaments [57]. The induced myogenic cells also displayed functional characteristics consistent with cardiac myocytes, such as rhythmic $Ca^{2+}$ fluctuations [57]. Another study not only showed that 5-azacytidine induces myocyte differentiation, but also that these induced cardiac myocytes can connect with adjoining cells and beat spontaneously 2 weeks later [58]. Additional *in vitro* experiments revealed the ability of MSCs to transdifferentiate into cardiomyocytes when co-cultured with other myocytes. Xu *et al.* showed that direct cell–cell contact is necessary for transdifferentiation to occur through formation of connexin-43-mediated gap junctions between bone-marrow-derived MSCs (BM-MSCs) and native myocytes. When cells were cultured in a dual chamber system separated by a semipermeable membrane, BM-MSCs failed to differentiate into cardiomyocytes, as noted by a lack of α-actinin expression. However, in a separate study, Li *et al.* showed that physical contact between cells is not necessary for cardiomyocyte differentiation [55]. Cumulatively, these findings suggest that cell–cell contact, the local microenvironment, and soluble factors secreted by both native myocytes and MSCs all play a role in the ability of MSCs to contribute to cardiomyogenesis.

*In vivo* experiments provide evidence of MSC engraftment as well as endogenous CSC proliferation/differentiation and resident cardiomyocyte cell signaling. In 2002, Toma *et al.* injected human MSCs transfected with β-galactosidase into the left ventricle through the diaphragm of immune-deficient mice and reported that at 4 days 0.44% of the injected cells could be found in the myocardium, while the majority of cells resided in the spleen, liver, and lungs [53]. While even fewer cells were found in the myocardium at 60 days postinjection, all the mice that survived expressed desmin, a marker of cardiac differentiation, as well as cardiac-specific troponin T, β-MHC, α-actinin, and phospholamban. In a swine model of chronic ischemic cardiomyopathy, Quevedo *et al.* further demonstrated the ability of MSCs to engraft and differentiate into cardiomyocytes [20]. In this study, female swine received either placebo or male allogeneic MSCs by transendocardial injections. Engraftment of MSCs was determined by co-localization of the Y chromosome (Ypos) and cardiomyocyte differentiation was determined by co-localization with GATA-4, Nkx2.5, and α-sarcomeric actin. Cell engraftment and subsequent differentiation were found in both

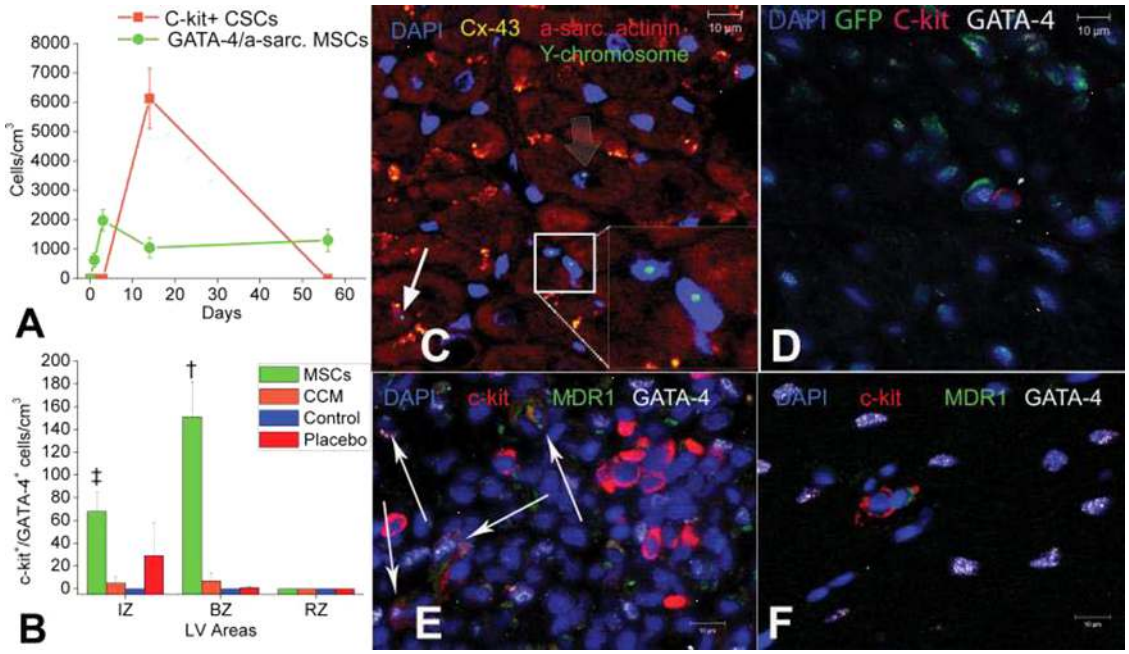



**Figure 45.3** Direct MSC stimulation of endogenous repair. (A) A graph depicting the contribution of cardiomyocyte precursors following exogenous administration of MSCs (green line) and endogenous CSCs (orange line), during cardiac repair after MI. MSC differentiation occurs rapidly after delivery. At 2 weeks, MSCs activate endogenous expansion of c-kit$^+$ CSCs (orange line). (B) Two weeks following transendocardial injection the number of c-kit$^+$ cells co-expressing GATA-4 is greater in MSC-treated hearts compared with non-MSC treated hearts. The cardiac precursors are preferentially located in the infarct zone (IZ) and border zone (BZ) of the MI, indicating an active process of endogenous regeneration (‡ $P = 0.019$ and †$P < 0.0001$). (C, D) The 2-week-old chimeric myocardium contains mature cardiomyocytes (open arrow), immature MSCs (arrowheads, inset), and cardiac precursors of MSC origin (arrow), coupled to host myocardium by connexin-43 gap junctions. Interestingly, endogenous c-kit$^+$ CSCs are found in close proximity to MSCs (D). (E) Cluster of c-kit$^+$ CSCs in an MSC-treated heart; numerous CSCs are committed to cardiac lineage documented by GATA-4 and MDR-1 co-expression (arrows). (F) A few isolated c-kit$^+$ cells were found in non-MSC-treated animals. (See color plate section.) *Source:* Hatzistergos *et al.* 2010 [4]. Reproduced with permission of American Heart Association.

the infarct and border zones of treated animals. Interestingly, engraftment correlated with increased contractile function and global left ventricular (LV) function in the infarct and border zones of treated animals compared with the placebo-treated group. These results demonstrated not only the ability of MSCs to engraft and differentiate into cardiomyocytes, but also their direct contribution to improved cardiac function.

Despite significant cardiac structural and functional improvements, the low rates of MSC engraftment and differentiation found in these studies have underscored the role of paracrine effects in cardiomyogenesis. However, a comparison of injection of allogeneic MSCs, concentrated conditioned medium (CCM) or placebo by Hatzistergos *et al.* highlighted the cell autonomous effects of MSC treatment in a porcine model of AMI. This study demonstrated that MSCs contributed to myogenesis by additional mechanisms, stimulation of endogenous CSC recruitment, proliferation, and myocardial lineage commitment (Figure 45.3) [4].

MSC-treated animals exhibited a 20-fold increase in c-kit$^+$ endogenous CSCs compared with those treated with CCM. This difference could be attributed to the ability of MSCs to form connexin-43-mediated gap junctions and N-cadherin mechanical connections with ckit$^+$ CSCs and adult cardiomyocytes as well as other MSCs. In addition to the stimulation of endogenous CSCs, MSC-treated animals exhibited a fourfold increase in cardiomyocyte turnover after MSC administration compared with CCM-treated animals as measured by the presence of the mitotic marker phospho-histone H3. These results indicated that while paracrine factor secretion does indeed play a distinct role in cardiomyocyte regeneration, MSC engraftment, differentiation, and interaction with the local milieu provides for a more complete recovery.

### 45.3.4 Neovascularization

Similar to the process of cardiomyogenesis, MSC-induced neovascularization is both multifaceted and synergistic. Possible mechanisms leading to vasculogenesis include the differentiation of MSCs into vascular smooth muscle cells and endothelial cells, the release of pro-angiogenic and pro-arteriogenic paracrine factors, as well as the recruitment of endothelial progenitor cells (EPCs). Several *in vitro* and *in vivo* studies have demonstrated the ability of MSCs to differentiate into endothelial and vascular smooth muscle cells [20,59,60]. However, substantial increases in perfusion, capillary density, and cardiac function, despite low rates of MSC engraftment, have highlighted the role of paracrine factor secretion in neovascularization. Vascular endothelial growth factor (VEGF), one of the first pro-angiogenic factors described, is of particular interest [61]. Several small and large

animal models of AMI have demonstrated increased VEGF expression in the myocardium of MSC-treated animals along with significant increases in angiogenesis, capillary density, tissue perfusion, and improvement in LV function [62,63]. Additionally, Markel *et al.* demonstrated that reducing VEGF in MSCs using small interfering RNA technology impaired both VEGF production and prevented MSC-mediated improvement in cardiac function following transplantation in a rat model of AMI [64]. However, while the administration of VEGF as a protein and as a DNA plasmid promotes angiogenesis, Shyu *et al.* showed that MSCs are superior to both angiopoietin-1 (Ang-1) and VEGF therapy in promoting cardiac regeneration [65]. Thus, MSC-induced neovascularization is mediated by the interaction of MSCs with multiple factors produced within the ischemic microenvironment. For example, NO, present after an AMI, promotes peripheral EPC mobilization to sites of injury and subsequent differentiation into mature endothelium [66]. In contrast, NO production inhibits MSC differentiation into endothelial cells [67] through a mechanism of upregulating VEGF receptor 2 present on EPCs, allowing VEGF-A to induce differentiation but downregulating the platelet-derived growth factor receptor on MSCs responsible for VEGF-A-induced endothelial cell differentiation. Therefore, it appears that the accumulation of NO in the inflammatory environment after MI allows for the recruitment of EPCs through VEGF-A production and subsequent vasculogenesis. After the release of anti-inflammatory factors by MSCs and the subsequent decrease in NO, MSC-mediated vasculogenesis can commence. Cumulatively, these results demonstrate the multifaceted nature of MSC-induced neovascularization and the need to prolong cell survival and engraftment in order to reap the full benefits of their use.

### 45.3.5 Paracrine effects

The robust cardiac structural and functional improvements demonstrated by MSC administration despite low MSC retention and engraftment rates have led to the hypothesis that MSCs secrete a variety of soluble factors, including cytokines, chemokines, and growth factors, which act both locally and systemically, leading to cardioprotective as well as cardioreparative effects. MSC-secreted proteins include those that promote angiogenesis, such as VEGF, stromal cell-derived factor 1 (also known as CXCL12), granulocyte colony-stimulating factor, TGF-β1, HGF, and Ang-1 [68,69]. Secreted pro-cardiogenic factors include BMP-2, HGF, TGF-β, and Jagged1 [5,70,71]. Those that promote cell homing and recruitment include thymosin-β4 and stromal cell-derived factor 1 [72,73]. Immunomodulatory factors include heme oxygenase-1, HGF, and IL-6 [73–75]. The antifibrotic actions of MSCs are mediated by MMP-1, -2 and, -9, as well as tumor necrosis factor-α [72]. Owing to the plethora of beneficial cardioprotective and regenerative effects generated by paracrine factor secretion, there has been an increased attempt to augment secretion of these factors through preconditioning, genetic modification, and pharmacotherapy.

### 45.3.6 Exosomes

Some of the cell autonomous paracrine effects of MSCs have been attributed to their release of exosomes or microvesicles derived from intracellular endosomes [76]. These membrane-bound vesicles range in size from 40 to 100 nm and can influence cellular activity through antigen presentation and the transfer of proteins, mRNA, and microRNA [77]. These characteristics allow exosomes to play an important role in a variety of cell processes, including, but not limited to, immune response, cell death, and angiogenesis [78–80]. Various studies have demonstrated a role for MSC-derived exosomes in tissue regeneration, including the treatment of acute kidney injury, liver fibrosis, hind limb ischemia, MI, and reperfusion injury [81–84]. In 2010 Lai *et al.* isolated a homogeneous population of 50–65 nm MSC-derived vesicles and were the first to demonstrate that these exosomes reduced infarct size in a mouse model of myocardial ischemia–reperfusion injury compared with saline-injected animals [85]. While the results attributed to exosomal therapy reveal a novel and exciting intercellular communication involved in MSC treatment, future studies are still needed to assess the safety and efficacy of MSC-derived exosomes in large animal models (see also Chapters 1, 20, and 38).

### 45.3.7 Mitochondrial transfer

In addition to the release of membrane-bound exosomes, MSCs have the capacity to transfer mitochondria between cells, thereby prolonging cellular lifespan and rescuing damaged cells by improving aerobic respiration. Spees *et al.* elegantly displayed the capacity for MSC-mediated mitochondrial transfer by co-culturing human MSCs with A549 $p^0$, cells that lack mitochondrial DNA and which are incapable of aerobic respiration due to pretreatment with ethidium bromide [86]. While aerobic respiration was restored and the functionality of the newly transferred mitochondria was determined, it was not understood whether the mitochondria had been transmitted as discrete vesicles or via direct cytoplasmic transfer. Various experiments have now provided compelling evidence that this process occurs through connexin-43-mediated gap junctions and actin-containing tunneling nanotubes [87,88]. Together, the evidence from exosome release and mitochondrial transfer experiments underscores the importance of MSC autonomous mechanisms of action and necessitates continued emphasis on the ability of MSCs to transfer therapeutic factors in a paracrine or direct cell-to-cell manner (see Chapters 10 and 21).

### 45.3.8 Preconditioning

The preconditioning of MSCs through stress induction, co-culture, and pharmacotherapy has emerged as a possible route to promote the cardioregenerative effects of MSCs. Preconditioning enhances paracrine factor secretion and improves cardiac structure and function in both small and large animal models and has already been the subject of clinical trials. Chacko *et al.* demonstrated that hypoxic preconditioning of MSCs in 0.5% oxygen for 24 or 48 h increased the expression of

pro-survival, pro-angiogenic, and pro-differentiation proteins [89]. Additionally, there was an upregulation of hypoxia-inducible factor-1α, a key angiogenic mediator, and phospho-AKT, a molecule involved in a cell signaling pathway that leads to cell survival by blocking apoptosis. Chacko *et al.* expanded on these results by demonstrating that multiple episodes of ischemic preconditioning of MSCs prior to longer periods of anoxia increased cell survival in a rat model of AMI compared with a single cycle of anoxia. Ischemic preconditioning significantly reduced apoptosis and increased cell engraftment through activation of AKT and hypoxia-inducible factor-1α pathways [89].

Co-culturing MSCs with various growth factors, including Ang-1 or a cocktail of growth factors (basic fibroblast growth factor, insulin-like growth factor (IGF)-1, and BMP2), increased cell survival, reduced scar size, and improved functional recovery in a rat model of AMI [90]. Interestingly, MSCs preconditioned with a cocktail of growth factors displayed enhanced connexin-43-mediated gap junction formation as well as increased cardiac-specific markers [5]. Finally, combined administration of simvastatin, a cardioprotective statin, in a swine model of AMI resulted in statistically significant improvements in MSC survival and differentiation compared with untreated MSCs, and this led to decreased fibrosis and improved cardiac function.

Statins, which are HMG-CoA reductase inhibitors, are a class of drugs used to lower cholesterol levels by inhibiting the enzyme HMG-CoA reductase, which plays a central role in the production of cholesterol in the liver. As indicated earlier, statins have also been found to prevent cardiovascular disease and mortality in those who are at high risk.

The improvements in cardiac structure and function described can be attributed to reduced inflammatory markers present in the local microenvironment of the swine hearts after infarction, providing an environment more conducive to cell engraftment, survival and differentiation [91].

### 45.3.9 Genetic modification

Genetic modification of MSCs has demonstrated promising results in various small and large animal models of ischemic cardiomyopathy. For example, several studies utilizing retroviral transduction of MSCs in order to overexpress the anti-apoptotic/cell survival gene *AKT* have demonstrated increased cell survival, engraftment, and cardiac function as well as reduced infarct size compared with control cells [92,93]. Gnecchi and colleagues overexpressed *AKT* in mouse or rat MSCs (Akt-MSCs) and showed that the administration of conditioned medium from hypoxia-preconditioned Akt-MSCs generated similar effects to the injection of the Akt-MSCs themselves, including significant reduction in fibrosis as well as restoration of ventricular function [73,94]. Studies suggest that a variety of paracrine factors mediate the therapeutic effect of Akt-MSCs, including secreted frizzled-related proteins 1 and 2 and IGF-1 [73,95,96]. *In vitro*, the Akt-MSC conditioned medium was able to induce spontaneous contraction of isolated rat cardiomyocytes. Additionally,

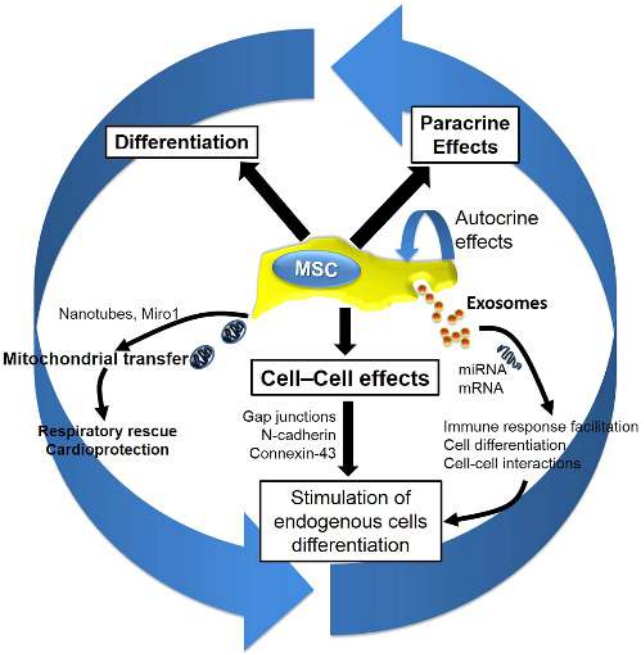



**Figure 45.4** MSC mechanisms of action. The proposed mechanism of action of MSCs forms an intertwined cycle of paracrine, autocrine, and direct effects that include vascular regeneration, myocardial protection, and cardiomyocyte regeneration that ultimately lead to cardiac action repair. *Source:* Karantalis and Hare 2015 [100]. Reproduced with permission of Wolters Kluwer Health.

expression of VEGF, fibroblast growth factor-2, HGF, IGF-1, and thymosin-β4 were all significantly upregulated in the Akt-MSCs compared with control MSCs. Combining Akt overexpression and another gene product has also proven effective. Shujia *et al.* showed that MSCs transduced to overexpress both Akt and Ang-1 led to increased engraftment and long-term survival, increased vascular density, and decreased infarct size compared with nontransduced MSCs and placebo in a rat model of AMI [97]. Furthermore, the transduced MSCs showed an increased capacity for vascular smooth muscle and cardiomyocyte differentiation, leading to improved LV function and reverse remodeling. Together, these and other results [98,99] suggest that genetic modification of MSCs is an exciting strategy to bolster the cardiac regenerative effects of MSCs, to circumvent the problem of poor cell survival in the proinflammatory post-infarction environment, and provide compelling evidence to move forward with additional preclinical studies with longer follow-up in order to ensure safety prior to clinical trials.

The many ways that MSCs mediate their therapeutic benefit are summarized in Figure 45.4.

## 45.4 Cell combination therapy

In addition to preconditioning and genetic modification, another novel approach to cell-based therapy involves cell combination therapy. As previously noted, *ex vivo* co-culturing of MSCs with

cardiomyocytes enhances MSC lineage commitment into a cardiac phenotype, and MSCs alone are able to stimulate proliferation and differentiation of endogenous CSCs *in vivo* [4]. These results prompted the exploration of combining MSCs and CSCs in hopes of capitalizing on the immunomodulatory and paracrine effects of MSCs while enhancing their differentiation capacity through interaction with CSCs. Williams *et al.* tested this approach in a swine model of AMI. Yorkshire swine were administered either a combination of human MSCs and human CSCs (200 million cells/1 million cells, $n = 5$) or human CSCs alone (1 million cells, $n = 5$), or human MSCs alone (200 million cells, $n = 5$) or placebo (phosphate-buffered saline, $n = 5$) by intramyocardial (transepicardial) injection [21]. One of the most exciting results of this study was the sevenfold increase in engraftment of the combination stem cell group compared with either cell group alone. Additionally, while all cell groups exhibited reduced infarct size, the antifibrotic effect was twofold greater in the cell combination group compared with the single cell groups. The combination cell group also showed the greatest improvement in both diastolic and systolic function, while all cell-treated animals exhibited similar improvements in LV ejection fraction (LVEF) and stroke volume compared with placebo-treated animals. Surprisingly, while both single cell-treated groups demonstrated a significant improvement in cardiac output in the combination group, the improvement was not significantly different from the placebo group. This study showed that combination stem cell therapy is safe and produces (in most cases) statistically significant improvements in cardiac structure and function compared with single cell therapy. This positive effect prompted its testing in a chronic model of ischemic cardiomyopathy using both autologous and allogeneic combination therapy without immunosuppressive agents. The autologous study has proven to be very promising, while the allogeneic preclinical trial is ongoing. Both studies compared a combination of MSCs and CSCs with either MSCs alone or placebo injection. While scar size reduced substantially with either cell treatment, functional improvements, including LVEF, cardiac output, and circumferential strain, were markedly greater in the combination-treated animals. These results have convinced the US Food and Drug Administration to approve a phase I clinical trial, The **C**ombination **O**f c-kit$^+$ cells and mesenchymal cells: a **N**ovel **C**ell study **E**valuating single and dual cell **R**egenerative **T**herapy in **C**hronic **H**eart **F**ailure Trial (The CONCERT-CHF Trial), that will be administrated by the National Institutes of Health-sponsored Cardiovascular Cell Therapy Research Network.

## 45.5 Clinical trials utilizing bone-marrow-derived mesenchymal stromal/stem cells

### 45.5.1 Acute myocardial infarction

The combination of safety and efficacy as determined from preclinical trials involving MSCs in both the acute and chronic settings of ischemic cardiomyopathy led to a swift transition into the clinical trial realm. While the exact mechanisms of action are not fully understood, the therapeutic potential of MSCs offers patients with heart failure, and a variety of other illnesses, the potential not only to halt but also to reverse their disease. The first clinical trial utilizing autologous human BM-MSCs for treatment of AMI was published in 2004 [101]. This groundbreaking study enrolled 69 patients who underwent primary percutaneous coronary intervention within 12 h after the onset of AMI. Patients were randomized, and 3 weeks after their balloon angioplasty/stenting they received intracoronary injections of either $8–10 \times 10^9$ autologous BM-MSCs or saline. This trial not only demonstrated safety, but also efficacy. Several imaging techniques were utilized to show significant improvements in LV hemodynamic measurements and cardiac functional indices between the two groups at 3 and 6 months follow-up. MSC-treated patients had a 14% increase in LVEF compared with placebo-treated patients at 3 months postinjection and exhibited an additional 10% increase at 6 months postinjection. Additionally, real-time cardiac electromechanical NOGA® mapping in the MSC group demonstrated significant improvements in cardiac mechanical capability 3 months after injection compared with pre-injection [101].

The NOGA system is a catheter-based, nonfluoroscopic, three-dimensional electromechanical mapping system that simultaneously registers the electrical and mechanical activities of the left ventricle, enabling online assessment of myocardial viability. The system distinguishes between viable, nonviable, stunned, and hibernating myocardium and can assess wall motion. By steering the deflectable mapping catheter tip gradually to the LV endocardial sites, the NOGA processing system reconstructs the shape of the left ventricle in three dimensions in real time.

In 2009, Hare *et al.* published the first study using allogeneic human BM-MSCs in a double-blinded, placebo-controlled, dose-ranging phase I study for the treatment of 53 patients with AMI [102]. Patients were randomized to receive an intravenous infusion of one of the three cell-doses ($0.5 \times 10^6$, $1.6 \times 10^6$ and $5.0 \times 10^6$ allogeneic human MSCs/kg) or vehicle 7–10 days after AMI. This study not only provided groundbreaking evidence that the intravenous infusion of allogeneic human MSCs was safe, but demonstrated efficacy as well. The MSC-treated group had an increased LVEF relative to baseline when measured at 3, 6, and 12 months after the injection. Pulmonary function, as assessed by forced expiratory volume in 1 s results, also improved in the MSC group but not in the placebo group. Lastly, fewer cardiac arrhythmias were reported in the MSC group, and overall clinical status at 6 months after treatment was improved. These encouraging results paved the way for additional allogeneic MSC clinical trials, resulting in equal numbers of trials using autologous or allogeneic MSCs by 2014. Additionally, the safety and efficacy of intravenous administration led to questions concerning the optimal mode of delivery.

### 45.5.2 Chronic myocardial infarction

The exciting results from clinical trials assessing the therapeutic potential of MSCs for AMI have encouraged researchers to focus on chronic MI, for which there are six published and/or ongoing clinical trials. A clinical trial by Mohyeddin-Bonab *et al.* was designed to assess the optimal mode of cell delivery. This trial was not only the first clinical pilot study of autologous MSCs for chronic MI but also sought to determine whether injection into the myocardium or intracoronary administration provided superior results [103]. Eight patients (mean age of 49 years) who had been living with heart failure for an average of 72 months received MSCs either by intramyocardial injection during coronary artery bypass grafting ($n = 5$) or by percutaneous intervention ($n = 3$). The control group consisted of eight matched patients who received the same treatment but without MSC injection. The study provided evidence that MSCs, regardless of the mode of injection, were safe, increased LVEF at 12–25 months (mean 18 months) after injection, improved New York Heart Association class, increased viable myocardium, and decreased infarction size at 6 months follow-up. However, the study did not provide data determining which mode of delivery was more beneficial, likely due to the small sample size. MSCs thus improve cardiac structure and function as well as quality of life for patients with chronic MI. While Mohyeddin-Bonab *et al.* demonstrated improvements in various cardiac parameters following MSC treatment, the concurrent administration of MSCs with coronary artery bypass grafting did not allow them to assess MSC autonomous effects.

The Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS) trial showed scar reduction, tissue perfusion and regional function improvements in patients with chronic ischemic cardiomyopathy who received autologous MSCs by intramyocardial injections into akinetic/hypokinetic nonrevascularized myocardial territories. Antifibrotic effects were seen in both the MSC-only treated segments as well as the revascularized-only segments. However, corresponding increases in viable tissue, improved regional contractility, and wall motion, as well as increased perfusion, were only seen in the MSC-treated group [104]. These improvements collectively contributed to a global increase in LVEF. Results from this study provide compelling evidence for MSC-induced neoangiogenesis, although whether it occurs through stimulation of endogenous CSCs or other resident cells, differentiation of MSCs into vascular smooth muscle/endothelial cells, or a combination of the two is unclear.

It is important to note that improvements in the previously mentioned parameters occurred to the greatest extent at the site of injection, suggesting that MSCs exert their greatest effects at the regional level, thus underscoring the significance of both the mode and the location of cell delivery.

Although the PROMETHEUS trial provided compelling evidence for the therapeutic application of MSCs in the clinical setting, the size of the trial and the lack of a placebo control were definite limitations. The Transendocardial Autologous Cells (human MSCs or BM-MNCs) in Ischemic Heart Failure Trial (TAC-HFT) trial, on the other hand, compared transendocardial injections of autologous MSCs, BM-MNCs, and placebo in 65 patients with chronic ischemic cardiomyopathy [105]. This study showed safety of delivery in all three groups, while only the MSC-treated and BM-MNC-treated patients displayed improvements in clinical measurements of quality of life using the Minnesota Living With Heart Failure (MLWHF) score and the 6 min walk test. Interestingly, infarct size as a percentage of LV mass was reduced and regional contractility improved in only the MSC-treated group. In contrast to the PROMETHEUS trial, LVEF was not increased by transendocardial MSC injections.

Another recent clinical trial by Hare and colleagues, The PercutaneOus StEm Cell Injection Delivery Effects On Neomyogenesis (POSEIDON) trial, not only compared allogeneic and autologous human BM-MSCs, but also explored the effect of cell dosage [106]. The 30 patients enrolled in this study were equally divided to randomly receive $2 \times 10^7$, $1 \times 10^8$, or $2 \times 10^8$ MSCs. Within each group of 10, five patients received autologous MSCs while the other five received allogeneic cells, although all patients underwent bone marrow aspiration. Interestingly, while there was an inverse relationship between cell dosage and improved cardiac structure and function, there was no significant difference between efficacy of allogeneic and autologous cells. This finding unveiled another important question about cell potency versus cell dosage.

Similar to the TAC-HFT trial, the POSEIDON trial utilized a transendocardial method of delivery. Using multidetector computed tomography and biplane left ventriculography, this study reported scar size reduction of approximately 44% in treated (allogeneic or autologous) segments versus a 25% reduction in nontreated segments [106]. Furthermore, while minimal scar reduction was evident in nontreated scar segments, an improvement in segmental LVEF was only seen in the MSC-treated segments. The primary endpoint for the TAC-HFT study was safety, which was satisfied, but while the lack of improved LVEF in the TAC-HFT trial is inconsistent with the similar improvements in LVEF in the PROMETHEUS and POSEIDON trials, the small sample size precludes definitive conclusions of efficacy.

Further proof of reverse remodeling in the POSEIDON trial is provided by the improved sphericity index (i.e., the ratio of the long and short axes of the left ventricle) in treated groups, as well as reduced diastolic and systolic volumes. However, end-diastolic volume and end-systolic volume reductions were only seen in the allogeneic-treated groups. In addition to the previously stated structural and functional improvements, the POSEIDON trial also reported improvements in New York Heart Association classification, 6 min walk tests, and MLWHF scores. Clinical improvements in treated patients, in addition to statistically significant evidence of reverse remodeling, further substantiate the role of MSCs in improving functional status and quality of life for patients with chronic ischemic cardiomyopathy.




**Figure 45.5** Twelve-lead electrocardiogram showing ST segment elevation (orange) in leads I, aVL, and V1–V5 with reciprocal changes (blue) in the inferior leads, indicative of an anterior wall MI. (See color plate section.) *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain and is attributed as follows: "12 Lead EKG ST Elevation tracing color coded" by Displaced – Own work. Licensed under Public Domain via Wikimedia Commons – http://commons.wikimedia.org/wiki/File:12_Lead_EKG_ST_Elevation_tracing_color_coded.jpg#mediavie.

Cumulatively, the results of these clinical trials demonstrate the safety and efficacy of MSCs as a therapeutic agent for acute and chronic ischemic cardiomyopathy.

## 45.6 Clinical trials utilizing adipose-derived mesenchymal stromal/stem cells

Adipose-tissue-derived MSCs have garnered attention not only due to their ease of isolation using a liposuction technique, but also because adipose tissue contains 500-fold more MSCs than adult bone marrow does [107], thereby reducing the time needed for cell expansion [108]. The vast array of tissues containing MSCs and the success of adipose-tissue-derived MSC transplantation in preclinical trials of acute ischemic cardiomyopathy [109] prompted swift transition to the clinical setting. In the Randomized Clinical Trial of Adipose-Derived Stem Cells in the Treatment of Patients With ST-elevation Myocardial Infarction (APOLLO trial), 14 patients with ST-segment elevation AMI were randomized 3 : 1 to receive intracoronary infusion of either $2 \times 10^7$ adipose-derived MSCs or placebo. Not only did this trial demonstrate the safety of adipose-derived MSCs for the first time in the acute setting of an MI, but it also provided evidence of a 50% infarct reduction, a significant improvement in perfusion defect, although there was no significant improvement in cardiac function. (A characteristic elevation of the ST segment of the electrocardiogram occurs in some cases of severe AMI, an example of which is shown in Figure 45.5.)

Together, the clinical trials described here that utilized both bone-marrow- and adipose-derived MSCs demonstrate safety and efficacy in the acute setting of an MI, necessitating additional and larger scale clinical trials.

The success of adipose-tissue-derived MSC transplantation in preclinical trials of chronic ischemic cardiomyopathy in addition to the APOLLO clinical trial prompted the Randomized Clinical Trial of Adipose-derived Stem Cells in Treatment of Non Revascularizable Ischemic Myocardium (PRECISE) clinical trial. In this study, adipose-derived MSCs were administered transendocardially to 21 patients with chronic ischemic cardiomyopathy, while six patients received placebo injections [108]. Results included improved $MVO_2$ values between groups from baseline to 6 and 18 months posttreatment, as well as significantly improved total LV mass measurements and wall motion score index values. Interestingly, neither scar size reduction nor increased LVEF were seen in either the APOLLO or the PRECISE trials. These results suggest that MSCs from different tissues are

not identical and produce their therapeutic effects by a different mechanism(s).

## 45.7 Preconditioning in the clinical setting

Promising results from a previously mentioned preclinical trial [110] prompted the Cardiopoietic Stem Cell Therapy in Heart Failure (C-CURE) trial. The underlying rationale and driving force for this study was that the induction of MSCs toward a cardiopoietic lineage should optimize repair potency [111]. The C-CURE trial ultimately enrolled 47 patients with chronic ischemic cardiomyopathy, 15 of whom received standard of care (control group) and 32 of whom received stromal cell injections. Autologous human BM-MSCs were harvested and isolated from the iliac crest of heart-failure patients followed by their *ex vivo* expansion. The MSCs were subsequently exposed to a cardiogenic growth factor cocktail to induce expression and nuclear translocation of certain cardiac transcription factors and thereby promote cardiopoietic lineage specification of the MSCs. The cells were further culture-expanded and the cardiopoietic cells were delivered by trans-endocardial injection. Cell-treated patients exhibited statistically significant increases in LVEF as well as decreased end-systolic volume values compared with patients treated with the standard of care. Additionally, the cell therapy group demonstrated significantly improved 6 min walk test values, but the MLWHF questionnaire results were not improved significantly. Along with the establishment of safety, these results provide evidence of improved cardiac structure and function and quality of life. As the first clinical trial to demonstrate the efficacy of lineage specification without the need for cardiac tissue harvesting, the C-CURE trial undeniably advances the field of cell therapy for heart disease. Further clinical validation is needed to compare nonlineage-specified MSC treatment as well as allogeneic lineage-specified cells.

## 45.8 Conclusions

MSCs have proven to be safe and effective in the treatment of acute and chronic heart disease. The unique immunomodulatory properties of MSCs and their secretion of a wide array of soluble factors sets them apart from other readily available or inducible cells. Additionally, preclinical and clinical trials demonstrating improved cardiac structure and function in the acute and chronic settings of ischemic cardiomyopathy encourage the eventual widespread use of MSCs as a therapeutic agent for the reversal of heart disease. Future clinical trials should address optimization of mode, location, and time of cell delivery, as well as the combination of different cells. Once preclinical trials establish the safety of using genetically modified MSCs, these experiments should move cautiously into the clinical realm. The future of MSC therapy lies in optimizing their efficacy, whether by cell survival, engraftment, secretion, and/or differentiation capacity.

## References


1 Go AS, Mozaffarian D, Roger VL, *et al.* (2014)Heart disease and stroke statistics – 2014 update: a report from the American Heart Association. *Circulation* **129**:e28–e292.

2 Lennon DP, Caplan AI (2006)Isolation of human marrow-derived mesenchymal stem cells. *Exp Hematol* **34**:1604–1605.

3 Spaggiari GM, Capobianco A, Abdelrazik H, *et al.* (2008)Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* **111**:1327–1333.

4 Hatzistergos KE, Quevedo H, Oskouei BN, *et al.* (2010)Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ Res* **107**:913–922.

5 Hahn JY, Cho HJ, Kang HJ, *et al.* (2008)Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. *J Am Coll Cardiol* **51**:933–943.

6 Jennings RB, Reimer KA (1991)The cell biology of acute myocardial ischemia. *Annu Rev Med* **42**:225–246.

7 Jennings RB, Murry CE, Steenbergen C, Jr, Reimer KA (1990) Development of cell injury in sustained acute ischemia. *Circulation* **82**:II2–II12.

8 Jennings RB, Steenbergen C, Jr, Reimer KA (1995)Myocardial ischemia and reperfusion. *Monogr Pathol* **37**:47–80.

9 Karsner HT, Saphir O, Todd TW (1925)The state of the cardiac muscle in hypertrophy and atrophy. *Am J Pathol* **1**:351–372.

10 Kajstura J, Leri A, Finato N, *et al.* (1998)Myocyte proliferation in end-stage cardiac failure in humans. *Proc Natl Acad Sci U S A* **95**:8801–8805.

11 Beltrami AP, Barlucchi L, Torella D, *et al.* (2003)Adult cardiac stem cells are multipotent and support myocardial regeneration. *Cell* **114**:763–776.

12 Mazhari R, Hare JM (2012)Translational findings from cardiovascular stem cell research. *Trends Cardiovasc Med* **22**:1–6.

13 Boudoulas KD, Hatzopoulos AK (2009)Cardiac repair and regeneration: the Rubik's cube of cell therapy for heart disease. *Dis Model Mech* **2**:344–358.

14 Wollert KC, Meyer GP, Lotz J, *et al.* (2004)Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet* **364**: 141–148.

15 Schachinger V, Erbs S, Elsasser A, *et al.* (2006)Improved clinical outcome after intracoronary administration of bone-marrow-derived progenitor cells in acute myocardial infarction: final 1-year results of the REPAIR-AMI trial. *Eur Heart J* **27**:2775–2783.

16 Traverse JH, Henry TD, Pepine CJ, *et al.* (2012)Effect of the use and timing of bone marrow mononuclear cell delivery on left ventricular function after acute myocardial infarction: the TIME randomized trial. *JAMA* **308**:2380–2389.

17 Traverse JH, Henry TD, Vaughan DE, *et al.* (2010)LateTIME: a phase-II, randomized, double-blinded, placebo-controlled, pilot trial evaluating the safety and effect of administration of bone

marrow mononuclear cells 2 to 3 weeks after acute myocardial infarction. *Tex Heart Inst J* **37**:412–420.
18 Traverse JH, Henry TD, Vaughan DE, *et al.* (2009)Rationale and design for TIME: a phase II, randomized, double-blind, placebo-controlled pilot trial evaluating the safety and effect of timing of administration of bone marrow mononuclear cells after acute myocardial infarction. *Am Heart J* **158**:356–363.
19 Perin EC, Willerson JT, Pepine CJ, *et al.* (2012)Effect of transendocardial delivery of autologous bone marrow mononuclear cells on functional capacity, left ventricular function, and perfusion in chronic heart failure: the FOCUS-CCTRN trial. *JAMA* **307**:1717–1726.
20 Quevedo HC, Hatzistergos KE, Oskouei BN, *et al.* (2009)Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc Natl Acad Sci U S A* **106**:14022–14027.
21 Williams AR, Hatzistergos KE, Addicott B, *et al.* (2013)Enhanced effect of combining human cardiac stem cells and bone marrow mesenchymal stem cells to reduce infarct size and to restore cardiac function after myocardial infarction. *Circulation* **127**:213–223.
22 Klyushnenkova E, Mosca JD, Zernetkina V, *et al.* (2005)T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. *J Biomed Sci* **12**:47–57.
23 Williams AR, Hare JM (2011)Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res* **109**:923–940.
24 Hare JM, Fishman JE, Gerstenblith G, *et al.* (2012)Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA* **308**:2369–2379.
25 Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970)The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* **3**:393–403.
26 Hass R, Kasper C, Bohm S, Jacobs R (2011)Different populations and sources of human mesenchymal stem cells (MSC): a comparison of adult and neonatal tissue-derived MSC. *Cell Commun Signal* **9**:12.
27 Meirelles LDS, Chagastelles PC, Nardi NB (2006)Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* **119**:2204–2213.
28 Caplan AI (1991)Mesenchymal stem cells. *J Orthop Res* **9**:641–650.
29 Segers VF, Lee RT (2008)Stem-cell therapy for cardiac disease. *Nature* **451**:937–942.
30 Dominici M, Le Blanc K, Mueller I, *et al.* (2006)Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* **8**:315–317.
31 Amado LC, Saliaris AP, Schuleri KH, *et al.* (2005)Cardiac repair with intramyocardial injection of allogeneic mesenchymal stem cells after myocardial infarction. *Proc Natl Acad Sci U S A* **102**:11474–11479.
32 Shake JG, Gruber PJ, Baumgartner WA, *et al.* (2002)Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* **73**:1919–1925.
33 Caparrelli DJ, Cattaneo SM, Bethea BT, *et al.* (2002)Pharmacological preconditioning ameliorates neurological injury in a model of spinal cord ischemia. *Ann Thorac Surg* **74**:838–844.
34 Woodbury D, Schwarz EJ, Prockop DJ, Black IB (2000)Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* **61**:364–370.
35 Le Blanc K, Tammik C, Rosendahl K, *et al.* (2003)HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* **31**:890–896.
36 Majumdar MK, Keane-Moore M, Buyaner D, *et al.* (2003)Characterization and functionality of cell surface molecules on human mesenchymal stem cells. *J Biomed Sci* **10**:228–241.
37 Ringdén O, Uzunel M, Rasmusson I, *et al.* (2006)Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* **81**:1390–1397.
38 Frangogiannis NG, Mendoza LH, Ren G, *et al.* (2003)MCSF expression is induced in healing myocardial infarcts and may regulate monocyte and endothelial cell phenotype. *Am J Physiol Heart Circ Physiol* **285**:H483–H492.
39 Mantovani A, Sozzani S, Locati M, *et al.* (2002)Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **23**:549–555.
40 Raffaghello L, Bianchi G, Bertolotto M, *et al.* (2008)Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* **26**:151–162.
41 Jiang XX, Zhang Y, Liu B, *et al.* (2005)Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* **105**:4120–4126.
42 Luz-Crawford P, Kurte M, Bravo-Alegria J, *et al.* (2013)Mesenchymal stem cells generate a $CD4^+CD25^+Foxp3^+$ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem Cell Res Ther* **4**:65.
43 Li G, Yuan L, Ren X, *et al.* (2013)The effect of mesenchymal stem cells on dynamic changes of T cell subsets in experimental autoimmune uveoretinitis. *Clin Exp Immunol* **173**:28–37.
44 Burr SP, Dazzi F, Garden OA (2013)Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance? *Immunol Cell Biol* **91**:12–18.
45 Corcione A, Benvenuto F, Ferretti E, *et al.* (2006)Human mesenchymal stem cells modulate B-cell functions. *Blood* **107**:367–372.
46 Rasmusson I, Le Blanc K, Sundberg B, Ringdén O (2007)Mesenchymal stem cells stimulate antibody secretion in human B cells. *Scand J Immunol* **65**:336–343.
47 Beltrami CA, Finato N, Rocco M, *et al.* (1994)Structural basis of end-stage failure in ischemic cardiomyopathy in humans. *Circulation* **89**:151–163.
48 Dobaczewski M, Bujak M, Zymek P, *et al.* (2006)Extracellular matrix remodeling in canine and mouse myocardial infarcts. *Cell Tissue Res* **324**:475–488.
49 Mias C, Lairez O, Trouche E, *et al.* (2009)Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction. *Stem Cells* **27**:2734–2743.
50 Tang J, Wang J, Guo L, *et al.* (2010)Mesenchymal stem cells modified with stromal cell-derived factor 1α improve cardiac remodeling via paracrine activation of hepatocyte growth factor in a rat model of myocardial infarction. *Mol Cells* **29**:9–19.
51 Shu T, Zeng B, Ren X, Li Y (2010)HO-1 modified mesenchymal stem cells modulate MMPs/TIMPs system and adverse remodeling in infarcted myocardium. *Tissue Cell* **42**:217–222.
52 Wakitani S, Saito T, Caplan A.I. (1995)Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* **18**:1417–1426.

53 Toma C, Pittenger MF, Cahill KS, *et al.* (2002)Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* **105**:93–98.

54 Li H, Yu B, Zhang Y, *et al.* (2006)Jagged1 protein enhances the differentiation of mesenchymal stem cells into cardiomyocytes. *Biochem Biophys Res Commun* **341**:320–325.

55 Li X, Yu X, Lin Q, *et al.* (2007)Bone marrow mesenchymal stem cells differentiate into functional cardiac phenotypes by cardiac microenvironment. *J Mol Cell Cardiol* **42**:295–303.

56 Xu M, Wani M, Dai YS, *et al.* (2004)Differentiation of bone marrow stromal cells into the cardiac phenotype requires intercellular communication with myocytes. *Circulation* **110**:2658–2665.

57 Xu W, Zhang X, Qian H, *et al.* (2004)Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype *in vitro*. *Exp Biol Med (Maywood)* **229**:623–631.

58 Makino S, Fukuda K, Miyoshi S, *et al.* (1999)Cardiomyocytes can be generated from marrow stromal cells *in vitro*. *J Clin Invest* **103**: 697–705.

59 Wang T, Xu Z, Jiang W, Ma A (2006)Cell-to-cell contact induces mesenchymal stem cell to differentiate into cardiomyocyte and smooth muscle cell. *Int J Cardiol* **109**:74–81.

60 Davani S, Marandin A, Mersin N, *et al.* (2003)Mesenchymal progenitor cells differentiate into an endothelial phenotype, enhance vascular density, and improve heart function in a rat cellular cardiomyoplasty model. *Circulation* **108**(Suppl 1):II253–II258.

61 Connolly DT (1991)Vascular permeability factor: a unique regulator of blood vessel function. *J Cell Biochem* **47**:219–223.

62 Tang YL, Zhao Q, Zhang YC, *et al.* (2004)Autologous mesenchymal stem cell transplantation induce VEGF and neovascularization in ischemic myocardium. *Regul Pept* **117**:3–10.

63 Halkos ME, Zhao ZQ, Kerendi F, *et al.* (2008)Intravenous infusion of mesenchymal stem cells enhances regional perfusion and improves ventricular function in a porcine model of myocardial infarction. *Basic Res Cardiol* **103**:525–536.

64 Markel TA, Wang Y, Herrmann JL, *et al.* (2008)VEGF is critical for stem cell-mediated cardioprotection and a crucial paracrine factor for defining the age threshold in adult and neonatal stem cell function. *Am J Physiol Heart Circ Physiol* **295**: H2308–H2314.

65 Shyu KG, Wang BW, Hung HF, *et al.* (2006)Mesenchymal stem cells are superior to angiogenic growth factor genes for improving myocardial performance in the mouse model of acute myocardial infarction. *J Biomed Sci* **13**:47–58.

66 Aicher A, Heeschen C, Mildner-Rihm C, *et al.* (2003)Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* **9**:1370–1376.

67 Gomes SA, Rangel EB, Premer C, *et al.* (2013)S-Nitrosoglutathione reductase (GSNOR) enhances vasculogenesis by mesenchymal stem cells. *Proc Natl Acad Sci U S A* **110**:2834–2839.

68 Noiseux N, Gnecchi M, Lopez-Ilasaca M, *et al.* (2006)Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation. *Mol Ther* **14**:840–850.

69 Caplan AI, Dennis JE (2006)Mesenchymal stem cells as trophic mediators. *J Cell Biochem* **98**:1076–1084.

70 Forte G, Minieri M, Cossa P, *et al.* (2006)Hepatocyte growth factor effects on mesenchymal stem cells: proliferation, migration, and differentiation. *Stem Cells* **24**:23–33.

71 Mohanty S, Bose S, Jain KG, *et al.* (2013)TGFβ1 contributes to cardiomyogenic-like differentiation of human bone marrow mesenchymal stem cells. *Int J Cardiol* **163**:93–99.

72 Kinnaird T, Stabile E, Burnett MS, *et al.* (2004)Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ Res* **94**:678–685.

73 Gnecchi M, He H, Noiseux N, *et al.* (2006)Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB J* **20**:661–669.

74 Chabannes D, Hill M, Merieau E, *et al.* (2007)A role for heme oxygenase-1 in the immunosuppressive effect of adult rat and human mesenchymal stem cells. *Blood* **110**:3691–3694.

75 Haynesworth SE, Baber MA, Caplan AI (1996)Cytokine expression by human marrow-derived mesenchymal progenitor cells *in vitro*: effects of dexamethasone and IL-1α. *J Cell Physiol* **166**:585–592.

76 Akyurekli C, Le Y, Richardson RB, *et al.* (2015)A systematic review of preclinical studies on the therapeutic potential of mesenchymal stromal cell-derived microvesicles. *Stem Cell Rev* **11**:150–160.

77 Lai RC, Chen TS, Lim SK (2011)Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* **6**:481–492.

78 Thery C, Zitvogel L, Amigorena S (2002)Exosomes: composition, biogenesis and function. *Nat Rev Immunol* **2**:569–579.

79 Robbins PD, Morelli AE (2014)Regulation of immune responses by extracellular vesicles. *Nat Rev Immunol* **14**:195–208.

80 Lee JK, Park SR, Jung BK, *et al.* (2013)Exosomes derived from mesenchymal stem cells suppress angiogenesis by down-regulating VEGF expression in breast cancer cells. *PLoS ONE* **8**:e84256.

81 Fouraschen SM, Pan Q, de Ruiter PE, *et al.* (2012)Secreted factors of human liver-derived mesenchymal stem cells promote liver regeneration early after partial hepatectomy. *Stem Cells Dev* **21**: 2410–2419.

82 Camussi G, Deregibus MC, Tetta C (2010)Paracrine/endocrine mechanism of stem cells on kidney repair: role of microvesicle-mediated transfer of genetic information. *Curr Opin Nephrol Hypertens* **19**:7–12.

83 Lai RC, Yeo RW, Tan KH, Lim SK (2013)Mesenchymal stem cell exosome ameliorates reperfusion injury through proteomic complementation. *Regen Med* **8**:197–209.

84 Zhang HC, Liu XB, Huang S, *et al.* (2012)Microvesicles derived from human umbilical cord mesenchymal stem cells stimulated by hypoxia promote angiogenesis both *in vitro* and *in vivo*. *Stem Cells Dev* **21**:3289–3297.

85 Lai RC, Arslan F, Lee MM, *et al.* (2010)Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* **4**:214–222.

86 Spees JL, Olson SD, Whitney MJ, Prockop DJ (2006)Mitochondrial transfer between cells can rescue aerobic respiration. *Proc Natl Acad Sci U S A* **103**:1283–1288.

87 Meinertz T, Lip GY, Lombardi F, *et al.* (2004)Trial finds sustained release propafenone increases time to recurrent atrial fibrillation compared with placebo. *Evid Based Cardiovasc Med* **8**:44–45, discussion 46–47.

88 Pasquier J, Guerrouahen BS, Al Thawadi H, *et al.* (2013)Preferential transfer of mitochondria from endothelial to cancer cells through tunneling nanotubes modulates chemoresistance. *J Transl Med* **11**:94.

89 Chacko SM, Ahmed S, Selvendiran K, *et al.* (2010)Hypoxic preconditioning induces the expression of prosurvival and proangiogenic markers in mesenchymal stem cells. *Am J Physiol Cell Physiol* **299**:C1562–C1570.

90 Liu XB, Chen H, Chen HQ, *et al.* (2012)Angiopoietin-1 preconditioning enhances survival and functional recovery of mesenchymal stem cell transplantation. *J Zhejiang Univ Sci B* **13**:616–623.

91 Yang YJ, Qian HY, Huang J, *et al.* (2009)Combined therapy with simvastatin and bone marrow-derived mesenchymal stem cells increases benefits in infarcted swine hearts. *Arterioscler Thromb Vasc Biol* **29**:2076–2082.

92 Mangi AA, Noiseux N, Kong D, *et al.* (2003)Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* **9**:1195–1201.

93 Lim SY, Kim YS, Ahn Y, *et al.* (2006)The effects of mesenchymal stem cells transduced with Akt in a porcine myocardial infarction model. *Cardiovasc Res* **70**:530–542.

94 Gnecchi M, He H, Liang OD, *et al.* (2005)Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nat Med* **11**:367–368.

95 Mirotsou M, Zhang Z, Deb A, *et al.* (2007)Secreted frizzled related protein 2 (Sfrp2) is the key Akt-mesenchymal stem cell-released paracrine factor mediating myocardial survival and repair. *Proc Natl Acad Sci U S A* **104**:1643–1648.

96 Dufourcq P, Descamps B, Tojais NF, *et al.* (2008)Secreted frizzled-related protein-1 enhances mesenchymal stem cell function in angiogenesis and contributes to neovessel maturation. *Stem Cells* **26**:2991–3001.

97 Shujia J, Haider HK, Idris NM, *et al.* (2008)Stable therapeutic effects of mesenchymal stem cell-based multiple gene delivery for cardiac repair. *Cardiovasc Res* **77**:525–533.

98 Tang J, Wang J, Zheng F, *et al.* (2010)Combination of chemokine and angiogenic factor genes and mesenchymal stem cells could enhance angiogenesis and improve cardiac function after acute myocardial infarction in rats. *Mol Cell Biochem* **339**:107–118.

99 Tang YL, Tang Y, Zhang YC, *et al.* (2005)Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *J Am Coll Cardiol* **46**:1339–1350.

100 Karantalis V, Hare JM (2015)Use of mesenchymal stem cells for therapy of heart disease. *Circ Res* **116**:1413–1430.

101 Chen SL, Fang WW, Ye F, *et al.* (2004)Effect on left ventricular function of intracoronary transplantation of autologous bone marrow mesenchymal stem cell in patients with acute myocardial infarction. *Am J Cardiol* **94**:92–95.

102 Hare JM, Traverse JH, Henry TD, *et al.* (2009)A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (Prochymal) after acute myocardial infarction. *J Am Coll Cardiol* **54**: 2277–2286.

103 Mohyeddin-Bonab M, Mohamad-Hassani MR, Alimoghaddam K, *et al.* (2007)Autologous *in vitro* expanded mesenchymal stem cell therapy for human old myocardial infarction. *Arch Iran Med* **10**:467–473.

104 Karantalis V, DiFede DL, Gerstenblith G, *et al.* (2014)Autologous mesenchymal stem cells produce concordant improvements in regional function, tissue perfusion, and fibrotic burden when administered to patients undergoing coronary artery bypass grafting: The Prospective Randomized Study of Mesenchymal Stem Cell Therapy in Patients Undergoing Cardiac Surgery (PROMETHEUS) trial. *Circ Res* **114**:1302–1310.

105 Heldman AW, DiFede DL, Fishman JE, *et al.* (2014)Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy: the TAC-HFT randomized trial. *JAMA* **311**:62–73.

106 Suncion VY, Ghersin E, Fishman JE, *et al.* (2014)Does transendocardial injection of mesenchymal stem cells improve myocardial function locally or globally? An analysis from the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis (POSEIDON) randomized trial. *Circ Res* **114**:1292–1301.

107 De Ugarte DA, Morizono K, Elbarbary A, *et al.* (2003)Comparison of multi-lineage cells from human adipose tissue and bone marrow. *Cells Tissues Organs* **174**:101–109.

108 Perin EC, Sanz-Ruiz R, Sanchez PL, *et al.* (2014)Adipose-derived regenerative cells in patients with ischemic cardiomyopathy: The PRECISE Trial. *Am Heart J* **168**:88–95. e82.

109 Valina C, Pinkernell K, Song YH, *et al.* (2007)Intracoronary administration of autologous adipose tissue-derived stem cells improves left ventricular function, perfusion, and remodelling after acute myocardial infarction. *Eur Heart J* **28**:2667–2677.

110 Behfar A, Yamada S, Crespo-Diaz R, *et al.* (2010)Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. *J Am Coll Cardiol* **56**:721–734.

111 Bartunek J, Behfar A, Dolatabadi D, *et al.* (2013)Cardiopoietic stem cell therapy in heart failure: the C-CURE (Cardiopoietic stem Cell therapy in heart failURE) multicenter randomized trial with lineage-specified biologics. *J Am Coll Cardiol* **61**:2329–2338.

CHAPTER 46

# The role of mesenchymal stem/stromal cells in the management of critical limb ischemia

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## 46.1 Introduction

Peripheral arterial occlusive disease, also commonly known as peripheral artery disease (PAD), is a manifestation of atherosclerosis caused by obstruction of peripheral arteries (Figure 46.1). Critical limb ischemia (CLI) is the severe subset and end stage of PAD and is characterized by severe pain at rest, nonhealing ischemic skin lesions, and, finally, gangrene of the extremity due to inadequate blood supply to the limb [1]. If no therapeutic intervention is made during this stage of the disease the inevitable outcome is limb amputation [2]. The blockage of arteries is primarily due to atherosclerosis.

Another cause of PAD, especially in Mediterranean and Asian countries, is thromboangitis obliterans, also known as Buerger's disease. This is a recurring progressive inflammation and thrombosis of small and medium arteries and veins of the hands and feet. It is thought to be an autoimmune disease that is strongly associated with use of tobacco products, primarily from smoking, but also from smokeless tobacco [3]. Buerger's disease was first reported by Felix von Winiwarter in 1879 in Austria, but it was not until 1908 that the disease was given its first accurate pathological description by Leo Buerger of Mount Sinai Hospital in New York City.

Other causes of PAD include other autoimmune disorders like systemic lupus erythematosus and acute conditions such as arterial embolism. The prevalence of PAD has been estimated to be around 27 million in Europe and North America [4]. It occurs in approximately 3–12% of the general population and increases with age [5–7]. Buerger's disease accounts for a variable proportion of patients with peripheral vascular disease throughout the world: 0.75% in North America, 0.5–5.6% in western Europe, 16–66% in Korea and Japan, 45–63% in India, and 80% in Israel among Jews of Ashkenazi descent [3,8]. Various risk factors causally related to PAD include diabetes mellitus, hyperlipidemia, hypertension, and smoking. Coexisting coronary artery disease and cerebrovascular disease are highly prevalent in patients with PAD, particularly in the elderly population. CLI, which is the most severe form of the disease, is seen in 1% of the general population [9].

Diagnostic studies include measurement of hemodynamic changes by ankle–brachial pressure index (ABPI) (Figure 46.2), transcutaneous partial oxygen pressure (TcPO2), toe brachial

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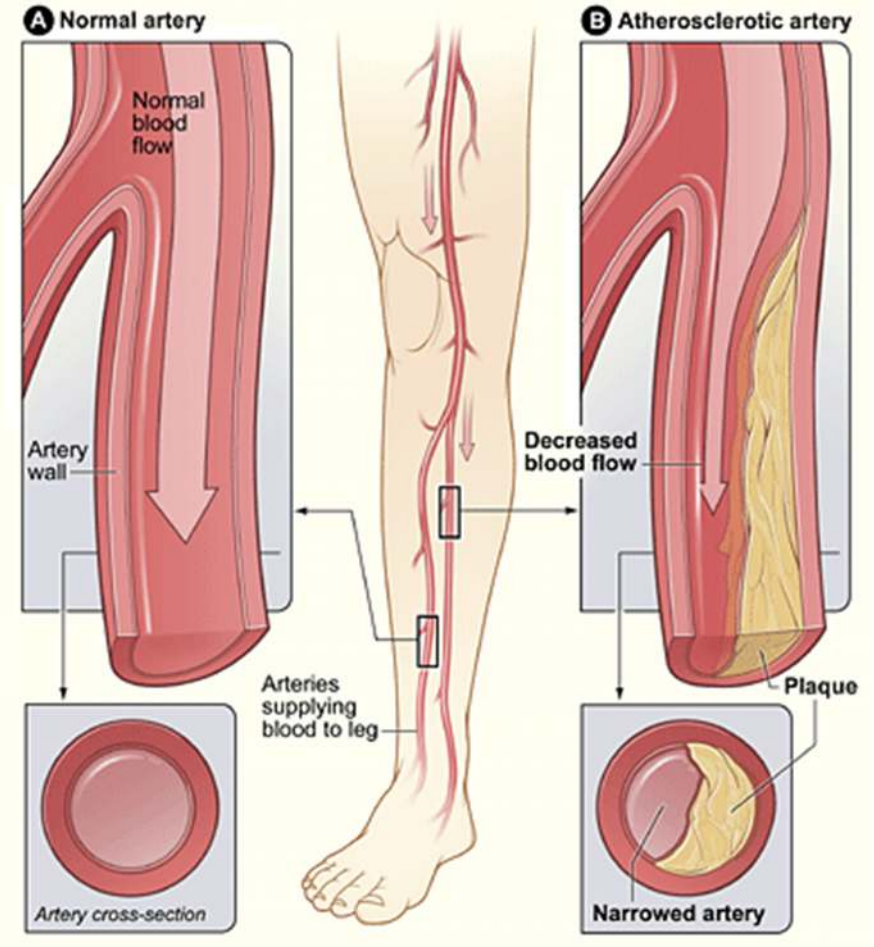



**Figure 46.1** The illustration shows how PAD can affect arteries in the legs. (A) A normal artery with normal blood flow. The inset image shows a cross-section of the normal artery. (B) An artery with plaque build-up that is partially blocking blood flow. The inset image shows a cross-section of the narrowed artery. Source: this image, reproduced here from Wikimedia Commons, is in the public domain. The author is the National Heart, Lung and Blood Institute, The National Institutes of Health, USA. Its attribution is as follows: http://www.nhlbi.nih.gov/health/health-topics/topics/pad/.

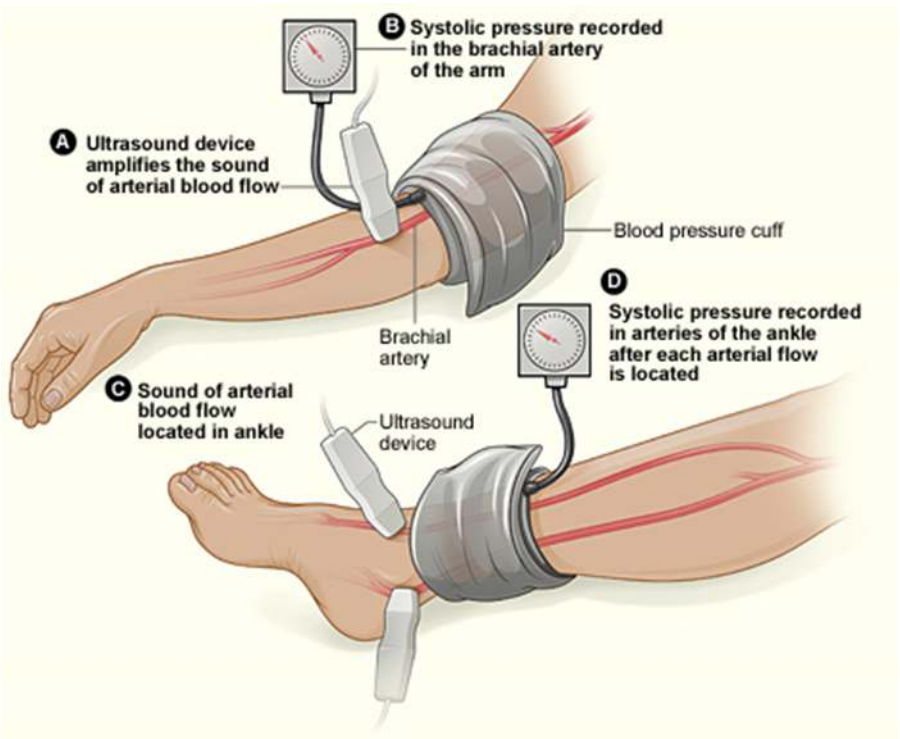



**Figure 46.2** The illustration shows the ankle–brachial index test. The test compares blood pressure in the ankle with blood pressure in the arm. As the blood pressure cuff deflates, the blood pressure in the arteries is recorded. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain. The author is the National Heart, Lung and Blood Institute, The National Institutes of Health, USA.

pressure index, exercise testing to elicit symptoms, segmental pressure monitoring, and Doppler examination of the vascular system.

Magnetic resonance angiogram and computed tomography angiography can also aid in the diagnosis [10]. The management of CLI is based on risk factor management and surgical or endovascular revascularization aiming to improve blood flow to the affected extremity [11]. Most patients with CLI are managed with analgesics and medications that inhibit platelet aggregation, such as cilostazol, in addition to lipid-lowering drugs. Cilostazol is a phosphodiesterase inhibitor with a therapeutic focus on cyclic adenosine monophosphate. It inhibits platelet aggregation and is a direct arterial vasodilator. Its main effects are dilation of the arteries supplying blood to the legs and decreasing platelet coagulation.

If revascularization has failed or is not possible, amputation is often necessary. Approximately 10% of CLI patients will undergo primary amputation, with healing rates varying between 30 and 90% and re-amputation rate at between 4 and 30%. Overall, approximately 40–50% of CLI patients will lose their leg within 6–12 months and approximately 15% will also require contralateral amputation within 2 years [12]. In CLI patients cardiovascular mortality increases substantially with approximately 20% of patients dying during the first 6 to 12 months after the onset of CLI, and 2, 5 and 10 year mortality rates of approximately 35%, 70% and 100% respectively [12]. It has therefore become urgent to develop new modalities of therapy that will induce revascularization, including neoangiogenesis and remodeling of the vascular system, to prevent the complications of CLI, thus improving limb salvage and increasing the overall survival of these patients.

## 46.2 Mesenchymal stem/stromal cells and angiogenesis

Several types of cells, particularly bone-marrow-derived mesenchymal stromal cells (BM-MSCs), have been shown to differentiate in the presence of appropriate growth stimuli along specific pathways including endothelial cells and perivascular cells. Mesenchymal stromal cells (MSCs) were isolated first from the bone marrow [13] and subsequently from a variety of other tissues, including adipose tissue, placenta, umbilical cord and cord blood, dental pulp, and amnion. MSCs are usually isolated by their plastic adherence property and can be expanded in large-scale culture for clinical use. Although no specific marker has been identified to isolate the MSC population, the International Society of Cell Therapy has defined these cells as being positive for CD73, CD105, CD90, and major histocompatibility complex class I antigens (human leukocyte antigen (HLA)-A, -B, and -C) and being negative for the hematopoietic markers CD45, CD34, CD14, CD19, and CD11b as well as major histocompatibility complex class II antigens (such as HLA-DR) [14]. The lack of a specific marker to identify MSCs has made it difficult to categorically determine the similarities or differences between the biological properties of these cells isolated from different tissues. Interestingly, BM-MSCs have been shown to possess several unique biological properties that are potentially beneficial for their use in both autologous and allogeneic cell therapy. Their intrinsic self-renewing ability and differentiation potential into chondrocytes, adipocytes, and osteocytes have been well documented [15,16].

Furthermore, MSCs are able to migrate and home to injured tissues, where they can act either by differentiating into cells of the musculoskeletal system if the injury is a musculoskeletal one and/or by secreting trophic factors that mediate paracrine signaling [16]. MSCs possess anti-inflammatory and immunomodulatory properties by a number of mechanisms, including inhibition of secretion of tumor necrosis factor-α (TNF-α) and interferon-γ and by increasing secretion of interleukin (IL)-10, indolamine 2,3 deoxygenase, prostaglandin $E_2$, and nitric oxide [17]. This unique immunomodulatory property makes these cells suitable for allogeneic, as well as autologous, use since they are nonimmunogenic as well as actively suppressive of T cells, B cells, natural killer cells, and macrophages and thus avoid rejection by the recipient's immune system [18].

It is now accepted that the myriad of growth factors and cytokines secreted by the MSCs *in vitro* and *in vivo* are critical for their therapeutic activity (Figure 46.3).

The major immunomodulatory cytokines secreted by MSCs include prostaglandin $E_2$, transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), stromal derived factor-1, nitric oxide, indolamine 2,3-deoxygenase, IL-4, IL-6, IL-10, IL-1 receptor antagonist, and soluble tumor necrosis factor-α receptor.

With regard to angiogenesis, MSCs secrete vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor, and angiopoietin (Ang)-1. These factors are released to recruit endothelial lineage cells and initiate vascularization [21]. MSCs also express mitogenic proteins such as TGF-α, TGF-β, HGF, epidermal growth factor, basic fibroblast growth factor, and IGF-1, which increase fibroblast, epithelial, and endothelial cell division [22]. These important factors are

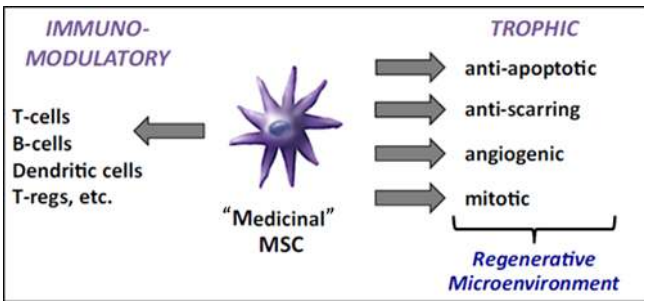



**Figure 46.3** The bioactive molecules secreted by medicinal MSCs are immunomodulatory and affect a variety of immune cell lineages [17]. Other secreted molecules establish a regenerative microenvironment by establishing a powerful trophic field [19]. *Source:* adapted from Caplan 2011 [20], pp. 11–15. Reproduced with permission of Elsevier.

known to play a key role in angiogenesis and act by a paracrine pathway [23,24]. Both VEGF and the angiopoietins (Ang-1 and Ang-2) play a crucial role in the process of neoangiogenesis. While Ang-1 and Ang-2 participate in the stabilization of the newly formed vessels, VEGF exerts its proangiogenic function by binding to one of its receptors, specifically VEGF receptor 2 (VEGFR2) also known as kinase insert domain receptor), which is expressed exclusively by endothelial cells and their precursors. This binding triggers a cascade of events leading to the formation of new blood vessels, including recruitment of support cells such as pericytes for small capillaries and smooth muscle cells for larger vessels [25]. MSCs have also been shown to possess anti-inflammatory, antiapoptotic, antifibrotic, mitogenic, and wound healing properties [26]. The anti-inflammatory properties of MSCs may be primarily responsible for the marked benefit of these cells in Buerger's disease, which is an inflammatory disease with a likely autoimmune basis affecting small and medium-sized arteries [27].

## 46.3 Potency assays for cells to be used in critical limb ischemia

The criteria for establishing the safety and quality of cellular therapies, both during production and at the time of lot release, are the use of potency assays to measure biologic activity appropriate to their intended clinical use. Potency testing, as stipulated by regulatory guidelines, should be a quantitative measure that reflects directly on the fundamental mechanistic pathway underlying the therapeutic response to verify the manufacturing process and is a measure of biological activity. A potency assay also provides the basis for comparability assessment after process changes, such as scale-up, site transfer, and new starting materials (e.g., a new MSC donor). Unlike defined component therapies with known molecular mechanisms, the therapeutic benefit of cellular therapies is often driven by its influence on a combination of mechanistic pathways and is often the result of a complex mixture of factors [28]. Cell viability is an important component of the potency of cell-based therapy. However, additional parameters of biological activity should also be tested [29] (and see Chapters 43 and 44).

Release specifications, which are acceptance criteria to be met by a product lot in order to allow administration to a patient, need to be defined in terms of identity, purity, and potency. Critical biological activity or potency for cell products can be defined by repeated testing of new cell lots in preclinical models. However, this is often not practical because the time taken for its execution is long and thus complicates lot release and clinical inventory requirements. Therefore, development of rapid, robust, consistent surrogate assays is required to ensure maintenance of biologic activity of cell therapies before their release for clinical use. Core requirements for potency testing as defined by the US Food and Drug Administration are presented in Table 46.1 [30].

**Table 46.1** US Food and Drug Administration core requirements for potency testing [30].

Potency testing of cell therapy product must:

- indicate product-specific biologic activity
- measure identity and activity of active component
- provide test results for product release
- provide data to establish stability specifications
- meet labeling requirements
- comply with biologics regulations and good manufacturing practice

Potency testing methods must:

- have predefined acceptance or rejection criteria
- include appropriate reference materials, standards, controls
- be amenable to validation
- have established and documented accuracy, sensitivity, specificity, and reproducibility
- provide quantitative data

Lehman *et al.* demonstrated that by using an *in vitro* endothelial tube formation assay a surrogate angiogenic potency assay could be developed that reflects the proangiogenic activity of multipotent adult progenitor cells (MAPCs) [28]. Serum-free conditioned media collected from MAPC culture induced endothelial tube formation. Three proteins, chemokine (C–X–C motif) ligand 5 (CXCL5), IL-8, and VEGF, were required and were linked to angiogenic potency. Depletion of any of these factors from the media prevented tube formation, while adding back increasing amounts of these cytokines into the depleted serum-free conditioned media established the lower limits of each of the cytokines required to induce angiogenesis. Thus, these markers were adopted as a surrogate potency assay for MAPC angiogenic activity.

### 46.3.1 Ixmyelocel-T

Ledford *et al.* demonstrated that ixmyelocel-T, which is an autologous patient-specific, expanded, multicellular therapy cultured from bone marrow mononuclear cells (BM-MNCs), contains a unique expanded population of M2-like macrophages that may limit tissue injury and promote repair [31]. It also contains large numbers of MSCs. M2 macrophages promote tissue repair and regeneration through various mechanisms, including immunomodulation and scavenging of tissue debris. It was proposed that delivering high numbers of these cells to ischemic tissues might limit tissue injury and promote repair. The authors demonstrated that macrophages in ixmyelocel-T therapy expressed the surface markers of M2 macrophages, CD206, and CD163. CD206 (also known as mannose receptor) is a 175 kDa type I membrane protein. It is a pattern recognition receptor belonging to the C-type lectin superfamily. CD163 is a scavenger receptor for the hemoglobin–haptoglobin complex.

After stimulation with lipopolysaccharide, they showed minimal secretion of the proinflammatory cytokines IL-12

and TNF-α compared with conventional M1 and M2 macrophages. Ixmyelocel-T macrophages efficiently ingested apoptotic BM-MNCs. They concluded that ixmyelocel-T therapy contained a unique population of M2-like macrophages that are characterized by expression of M2 markers, decreased secretion of proinflammatory cytokines after inflammatory stimuli, and efficient removal of apoptotic cells and that they may have a potential role in tissue repair and regeneration.

### 46.3.2 Stempeucel®

Stempeutics Research in Bangalore has developed a cell product called stempeucel®, which is an adult human bone-marrow-derived, cultured, pooled allogeneic MSC population. The cells show greater than 90% positivity for MSC-associated markers CD73, CD90, and CD105 and do not express CD34, CD45, or HLA-DR or co-stimulatory molecules (Figure 46.4) [32,33].

The probable mechanism of action of stempeucel in CLI is through secretion of growth factors, chemokines, and cytokines that are known for their pro-angiogenic, anti-inflammatory, and immunomodulatory functions [34]. BM-MSCs are known to secrete angiogenic cytokines such as VEGF, IL-6, IL-8, and Ang-1, and they have been shown to be potently angiogenic [35]. VEGF has a unique feature compared with the other growth factors in the regulation of new blood vessel formation in that it has a high degree of specificity for endothelial cells. VEGF treatment of cultured endothelial cells promotes cell survival, proliferation, migration, calcium influx, and branching morphogenesis [36]. In order to understand the exact mechanisms involved, stempeucel was initially screened for the expression of angiogenic genes such as VEGF A and its receptor VEGFR2, IL-6, Ang-1, HGF, keratinocyte growth factor (KGF), IGF-1, and TGF-β by reverse transcriptase polymerase chain reaction (Figure 46.5). The results clearly demonstrated that stempeucel expressed most of the angiogenic genes and that VEGF could potentially be a surrogate potency marker for stempeucel [31–33].

A possible potency assay for an MSC-based product for use in CLI could be its ability to initiate the process of neoangiogenesis. The potency assay could indicate the mechanism of action of the cellular product and could be used for quality control (stability) throughout the manufacturing process. Different studies [28,31] have demonstrated that various surrogate angiogenic markers can be established to demonstrate the potency of the product in CLI. These include VEGF, CXCL5, IL-8, endothelial tube formation, markers of M2 macrophages (CD206, CD163), IL-6, Ang-1, HGF, KGF, IGF-1, and TGF-β.

Selection of an angiogenic marker or markers is important in order to demonstrate the ability of the product to effect the desired therapeutic result. Developing at least two different methods increases the chances that one of them will be suitable for the product and will satisfy the requirements of the regulatory authorities [37].

## 46.4 Preclinical studies

### 46.4.1 Preclinical safety studies

Many studies have demonstrated that MSCs are nontoxic and nontumorigenic when tested in various preclinical animal models. MSCs from different tissue sources have been studied for potential side effects according to the regulatory criteria of respective countries (see Chapter 44). Studies involving both allogeneic and xenogeneic MSCs have shown that these cells are nontoxic. Similar results have been found in numerous clinical trials. Ra *et al.* studied the safety of human adipose-tissue-derived MSCs (ADMSCs) in humans and animals [38]. Immunodeficient mice were exposed to different doses of human ADMSCs intravenously and were observed for 13 weeks. There were no side effects and no mortality was observed. Tumorigenicity testing in BALB/c-nu (nude) immunodeficient mice showed no evidence of tumor formation even at the highest cell dose used.

Isakova *et al.* injected BM-MSCs into the central nervous system of young adult rhesus macaque monkeys to evaluate their safety and feasibility as vectors for direct intervention in neurologic disorders. The study demonstrated that the procedure of cell implantation was well tolerated and there was no observed toxicity attributable to the injected MSCs. Importantly, unlike the study by Ra *et al.*, in which xenogeneic cells were used, the study by Isakova *et al.* used syngeneic MSCs isolated from the iliac crest of a male rhesus macaque [39].

There have been concerns over the possibility of aggregation of these cells producing emboli in the lungs [40]. However, Eggenhofer *et al.* demonstrated the MSCs delivered by intravenous infusion have a short life and do not migrate to other organs [41], although other studies of intravenously injected MSCs have shown that after initial lodgment in the lungs they can subsequently migrate to sites of inflammation beyond the pulmonary capillary bed (see Chapters 22 and 23).

We have also conducted preclinical safety studies using BM-MSCs. These studies included acute toxicity studies (14 days single dose and 14 days repeat dose) by intravenous and intramuscular administration of stempeucel, as well as longer toxicity studies (90 days single dose) in two animal species (rats and rabbits) by two routes (intravenous and intramuscular) of administration. The toxicity studies were conducted using human-equivalent doses in respective animals to a maximum dose of $20 \times 10^6$ BM-MSCs/kg body weight. In addition, tumorigenicity and prenatal developmental toxicity studies have also been performed in immunocompromised mice and Sprague Dawley rats respectively. Results have shown that these cells are safe, nontumorigenic, nonteratogenic, and did not induce genotoxicity (Mathi *et al.* unpublished results).

Studies using a wide range of doses ($2 \times 10^6$ cells/kg body weight to $250 \times 10^6$ cells/kg body weight) of MSCs have been used in preclinical studies and have not shown signs of toxicity in rodents and large animal studies, including subhuman primates.

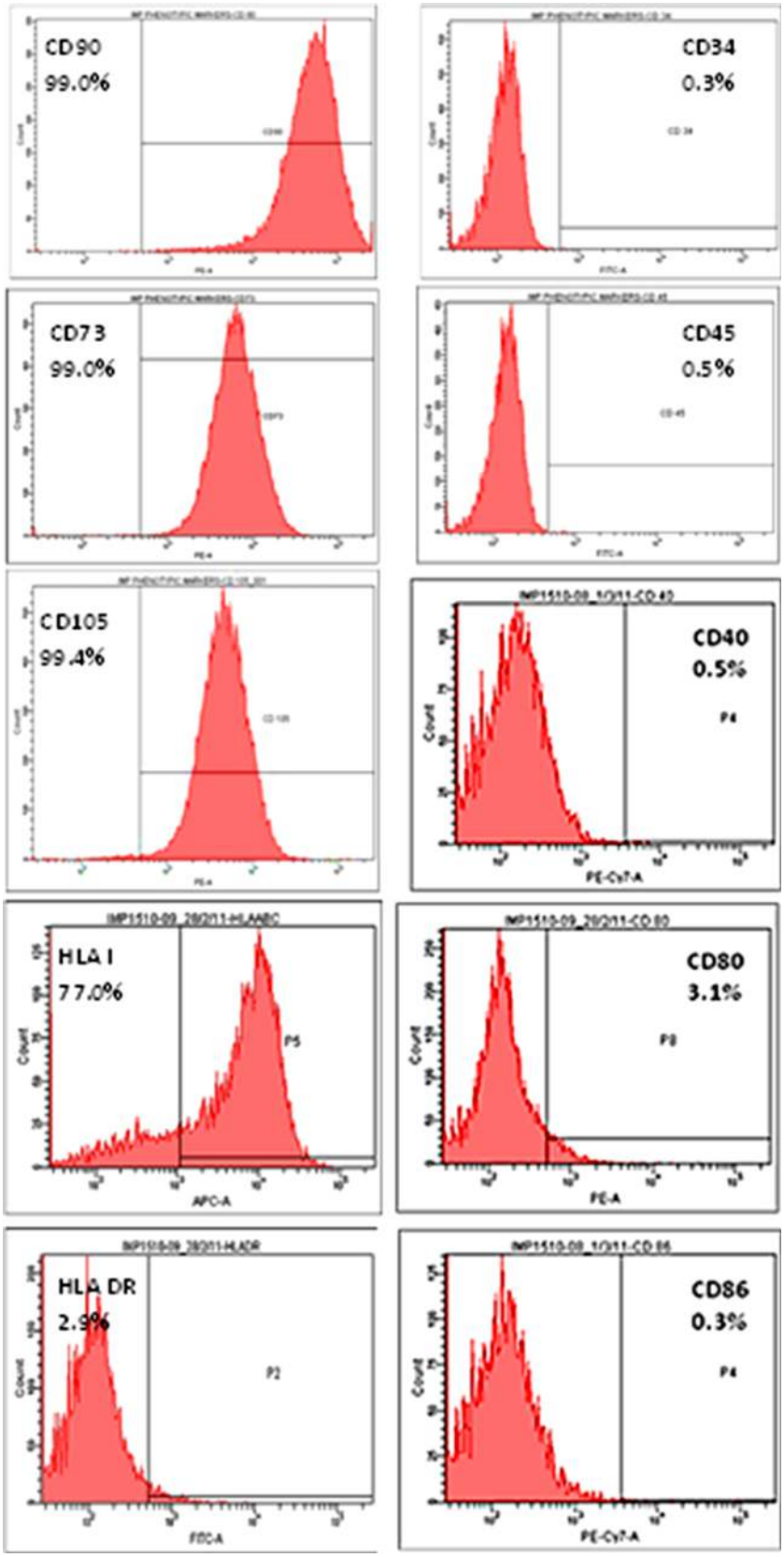



**Figure 46.4** Immunophenotype analysis of stempeucel. Stempeucel is positive for expression of the characteristic mesenchymal stromal cell markers CD90, CD73, and CD105 and negative for hematopoietic markers CD34 and CD45, as well as HLA-DR and the immune co-stimulatory markers CD80 and CD86.

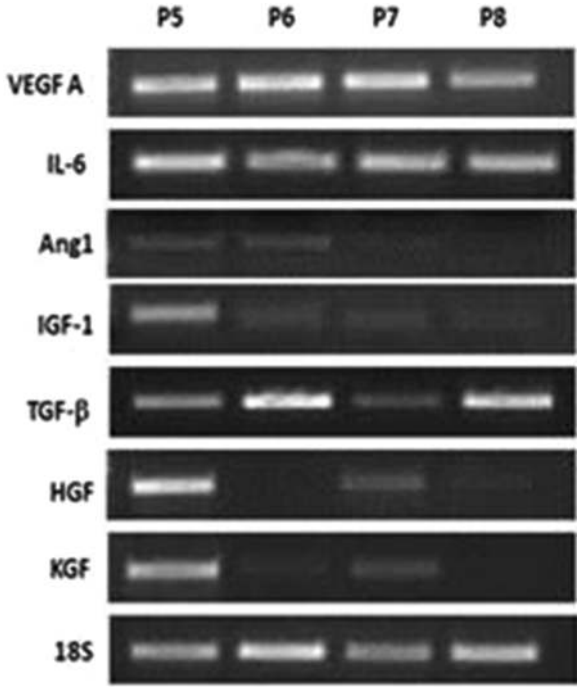



**Figure 46.5** Reverse transcriptase polymerase chain reaction showing that stempeucel expresses most of the angiogenic genes, including VEGF A, IL-6, Ang-1, IGF-1, TGF-β, HGF, and KGF from passage 3 to passage 7.

### 46.4.2 Preclinical efficacy studies

It is also important to demonstrate efficacy of any cell therapy product in an animal model of disease before administering the same cells to humans with the same disease. Unlike conventional small molecules, stem cell products face a unique challenge in this area. The disparity lies in determining the suitability of using animal stem cells in animals or human stem cells in immunocompromised/immunocompetent animals. A common regulatory requirement is to have animal data for the same test product that is intended to be tested in humans. This infers a regulatory preference for testing a human cell product in an animal disease model that closely resembles the human disease condition for which the product is intended. However, to validate the concept of efficacy, scientists have more commonly tested animal cells in animals.

In the context of this chapter, numerous animal models have shown that transplantation of MSCs or mononuclear cells (MNCs) from different sources augmented arteriogenesis in the ischemic limb of the animal [42,43]. Kim *et al.* compared the angiogenic potency of human mesenchymal stem/stromal cells derived from adipose tissues (ADSC) and bone marrow in a nude mouse model of hind limb ischemia [42]. Two weeks after transplantation the laser Doppler perfusion index was significantly higher in the ADSC group and BM-MSC groups compared with animals in the control group. The authors demonstrated that human MSCs can be a potential source for therapeutic angiogenesis.

Iwase *et al.* studied whether MSCs or MNCs would be a better option for treatment of CLI in a rat model of hind limb ischemia [43]. Three weeks after injection the laser Doppler perfusion index was noted to be significantly higher in the MNC group than in the control group. Capillary density was found to be higher in the MSC group than in the MNC-treated group. The numbers of endothelial cells and vascular smooth cells derived from the injected cells were also higher in the MSC group than in the MNC group. The study concluded that MSCs provided greater improvement in hind limb ischemia than MNCs.

We have performed preclinical efficacy studies in an animal model of hind limb ischemia in BALB/c nude mice to determine the efficacy of stempeucel. We used an extended femoral ligation method as the model and injected stempeucel intramuscularly at two different doses around the ligation. In the control group, all animals developed foot necrosis, whereas in the stempeucel group the highest dose of $5 \times 10^6$ MSC-treated animals showed 43% protection from foot necrosis. The results clearly demonstrated that animals treated with the highest dose of stempeucel had a better outcome than animals treated with the lower dose.

Based on this preliminary study a second efficacy study was performed with stempeucel at a dose of $5 \times 10^6$ cells. The results clearly showed a significant improvement in limb function, limb salvage, and histology scores, including muscle fiber area in animals treated with stempeucel, compared with the animals treated with the vehicle control (unpublished results). Histological analysis of limb muscles at day 28 after stempeucel injection revealed significant increase in the total muscle fiber area and significantly reduced muscle degeneration, inflammation, and muscle necrosis (Figure 46.6).

These findings suggest that allogeneic human BM-MSCs have the therapeutic potential to reduce inflammation and muscle degeneration and to promote neoangiogenesis for the healing of ischemic ulcers in CLI patients.

## 46.5 Clinical trials in critical limb ischemia

### 46.5.1 Safety of mesenchymal stromal cells in clinical trials

Lalu *et al.* conducted a systematic review of the literature and a meta-analysis of clinical trials to evaluate the safety of MSCs given intravenously or intra-arterially to humans [44]. A total of 1012 participants with various clinical conditions were included. Eight studies were randomized controlled clinical trials and enrolled 321 of the total 1012 patients studied. Both adult populations and mixed adult and pediatric populations were included. Studies using differentiated MSCs or additional cell types were excluded. There was a significant association between MSCs and transient fever at or shortly after MSC administration, which was not associated with long-term sequelae. Importantly, the pooled analysis recorded no serious adverse events due to administration of the MSCs and specifically found no association between MSCs and tumor formation. Other potential risks, such

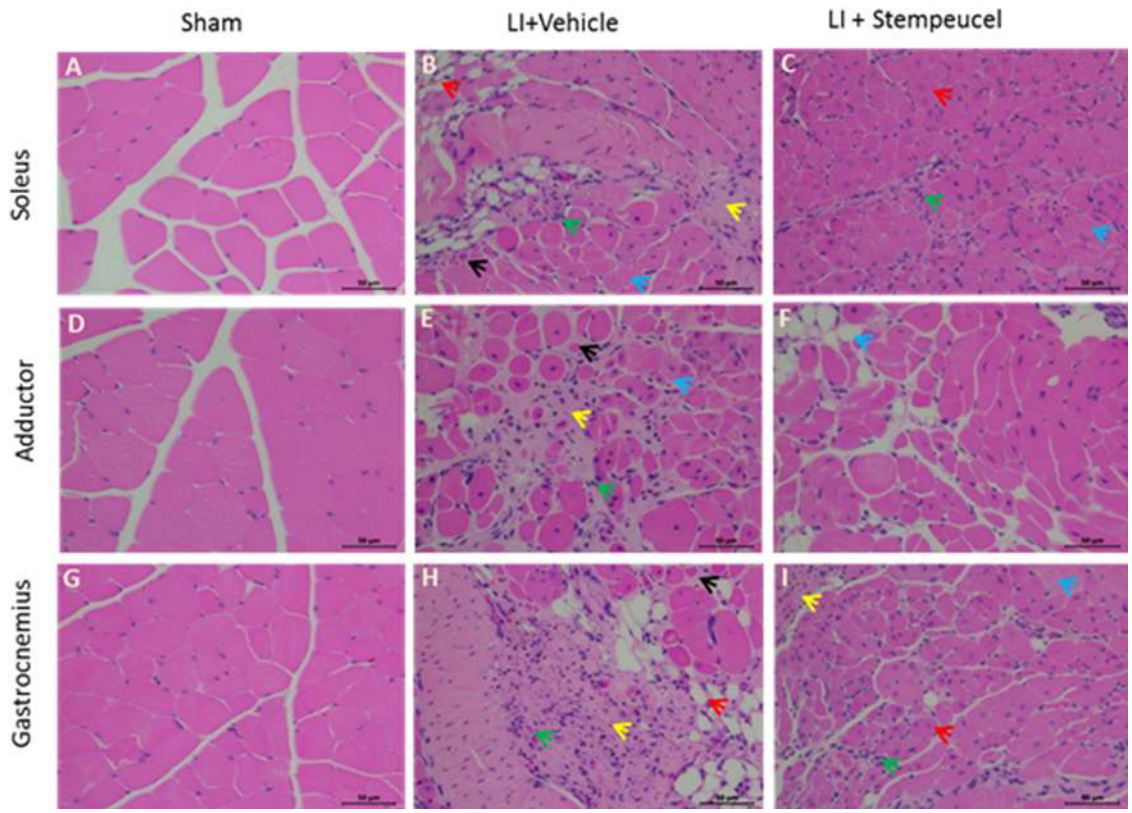



**Figure 46.6** Histology (hematoxylin and eosin stain) of sham-treated, arterial ligation+vehicle and arterial ligation+stempeucel animals after 28 days. (A, D, G) Cross-sections of skeletal muscles (A; soleus; D: adductor; G: gastrocnemius) of sham-treated animals showing the normal histology of muscle fibers with a peripheral nucleus arranged in muscle bundles with occasional blood vessels seen in between. (B, E, H) Cross-sections of the skeletal muscles (B; soleus; E: adductor; H: gastrocnemius) of animals after arterial ligation treated with vehicle showing severe vacuolar degeneration (red arrow), infiltration of inflammatory cells (green arrow), necrosis of muscle fibers (yellow arrow), degeneration of muscle fibers (blue arrow), and atrophy of muscle fibers (black arrow). (C, F, I) Cross-sections of the skeletal muscles (C; soleus; F: adductor; I: gastrocnemius) after arterial ligation in animals treated with stempeucel showing very mild degeneration of muscle fibers (blue arrow), infiltration of inflammatory cells (green arrow), and necrosis of muscle fibers (yellow arrow). Many regenerating muscle fibers (red arrow) with a centrally placed nucleus are observed. (See color plate section.)

as acute infusional toxicity, organ system complications, infection, or death, were also not associated with MSC administration. Hence, one can conclude that systemic administration of MSCs is safe.

### 46.5.2 Efficacy of mesenchymal stromal cells in clinical trials of critical limb ischemia

Several clinical trials have been conducted using AD- or BM-MSCs in CLI, both in atherosclerotic PAD and in Buerger's disease. Administration has been fairly standardized, with cells being administered in most studies by intramuscular injection into the gastrocnemius muscle. A few studies have also tried intra-arterial injections. There has been a preference for administering the MSCs locally around the ischemic ulcers for better healing.

On searching clinicaltrials.gov using the keywords "stem cells" and "CLI" we found 46 completed or ongoing clinical trials. Fifteen studies were using mesenchymal stem/stromal cells as the investigational agent, while other studies were using autologous MNCs from the bone marrow. TACT (Therapeutic Angiogenesis using Cell Transplantation) was the first large report on the use of BM-MNCs in the treatment of CLI [45]. In this pilot study they investigated the efficacy and safety of autologous implantation of BM-MNCs in patients with ischemic limbs because of PAD. They concluded that administration of autologous BM-MNCs was safe and effective for obtaining therapeutic angiogenesis, presumably because of the ability of marrow-derived cells to supply endothelial progenitor cells and to secrete various angiogenic factors or cytokines. They further suggested that the success of BM-MNCs transplantation was due to a higher number of $CD34^+$ hematopoietic stem cells and immature precursor cells in the bone marrow compared with peripheral blood. This was the first study using cells for the treatment of limb ischemia, and it was followed by a number of studies across the globe. The studies published in clinicaltrials.gov using mesenchymal stem cells are shown in Table 46.2.

Idei *et al.* assessed long-term clinical outcomes after BM-MNC administration in 51 patients with CLI, including 25 patients with PAD and 26 patients with Buerger's disease. Forty-six CLI

**Table 46.2** Clinical trials using MSCs in CLI registered with clinicaltrials.gov.

| | Type of cells | Condition | Trial phase | Enrolment | Primary outcome measures | Sponsor or collaborators | NCT number |
|---|---|---|---|---|---|---|---|
| 1 | MNCs and MSCs | CLI | Phase II | 50 | Change in angiogenesis<br>Change in blood supply | National University of Malaysia<br>Cytopeutics PTL | NCT01456819 |
| 2 | MSCs | CLI | Phase I<br>Phase II | 20 | Adverse events and symptomatic relief | Stempeutics Research Pvt Ltd | NCT00883870 |
| 3 | BM-MSCs | CLI/Buerger's disease | Phase II | 126 | Relief of rest pain<br>Healing of ulcerations or reduction of ulcer area in the target limb | Stempeutics Research Pvt Ltd | NCT01484574 |
| 4 | ADMSCs | CLI/diabetes mellitus | Phase I<br>Phase II | 33 | Angiographic assessment of neovasculogenesis (angiogenesis plus arteriogenesis).<br>Major adverse events (death, target limb amputation) | Iniciativa Andaluza en Terapias Avanzadas - Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas | NCT01257776 |
| 5 | MSCs and MNC | Autologous transplantation. Diabetic foot | Phase I | 40 | Magnetic resonance angiography | Third Military Medical University | NCT00955669 |
| 6 | UCMSCs | Diabetic foot/ CLI | Phase I<br>Phase II | 50 | Angiographic evaluation of angiogenesis at ischemic limb.<br>Pain | Qingdao University | NCT01216865 |
| 7 | ADMSCs | CLI | Phase I<br>Phase II | 20 | Major adverse events | K-Stemcell Co Ltd/Pusan National University Hospital | NCT01663376 |
| 8 | BM-MSCs | CLI/PAD | Phase I<br>Phase II | 30 | Collection of adverse events<br>Safety laboratory values<br>ECG findings<br>Analysis of inflammation markers | Apceth GmbH & Co. KG | NCT01351610 |
| 9 | ADMSCs | CLI | Phase I/ phase II | 33 | Number of adverse events and serious adverse events | Iniciativa Andaluza en Terapias Avanzadas – Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas | NCT01745744 |
| 10 | ADMSCs | CLI | Phase I<br>Phase II | 60 | Safety | Kasiak Research Pvt Ltd | NCT02145897 |
| 11 | BM-MSCs | Diabetic foot | Phase II | 30 | Survival. Major amputation. Primary wound healing. Ipsilateral relapse | Ruhr University of Bochum | NCT01065337 |
| 12 | ERCs | Peripheral vascular diseases | Phase I<br>Phase II | 15 | Safety | Medistem Inc. | NCT01558908 |
| 13 | ADMSCs | Non-revascularizable critical ischemia of the lower limbs | Phase I<br>Phase II | 10 | Safety | Instituto de Investigación Hospital Universitario La Paz/ Hospital Universitario La Paz | NCT01824069 |
| 14 | BM-MSCs | Type I diabetes mellitus with ulcer/type II diabetes mellitus with ulcer | Phase I<br>Phase II | 10 | Adverse events | Sheba Medical Center | NCT01686139 |

(*continued*)

**Table 46.2** (*Continued*)

| | Type of cells | Condition | Trial phase | Enrolment | Primary outcome measures | Sponsor or collaborators | NCT number |
|---|---|---|---|---|---|---|---|
| 15 | BM-MSCs | Critical ischemia | Phase I<br>Phase II | 48 | Serious adverse events | Iniciativa Andaluza en Terapias Avanzadas – Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas | NCT02287974 |
| 16 | UCMSCs | Diabetes mellitus/PAD | Phase I<br>Phase II | 30 | Angiographic evaluation of angiogenesis | Institute of Hematology & Blood Diseases Hospital | NCT02287831 |
| 17 | Combination cell therapy with MSCs and EPCs | CLI/severe leg ischemia/PAD/ peripheral vascular disease | Phase II | 35 | Enhancement of vessel formation accessed by nuclear perfusion scan | TCA Cellular Therapy | NCT00721006 |
| 18 | Combination cell therapy | CLI/peripheral vascular disease | Phase I | 10 | Safety | TCA Cellular Therapy | NCT00518401 |
| 19 | Expanded autologous adipose-derived adult stroma/stem cells | Peripheral vascular diseases/ cardiovascular diseases | Phase I<br>Phase II | 13 | Adverse events and tolerability | University Hospital, Toulouse/Unity Research 5241 UPS/CNRS/French Blood Establishment (Midi-Pyrénées)/Clinical Investigation Centre | NCT01211028 |
| 20 | Peripheral blood-derived autologous angiogenic cell precursors | CLI | Phase II | 22 | Safety<br>Rest pain<br>Pain-free walking distance<br>Ulcer size<br>Gangrene dimension and intensity<br>Improvement of tissue perfusion | Salus Ltd/TheraVitae Ltd | NCT01584986 |

MNCs: mononuclear cells; MSCs: mesenchymal stromal cells; BM-MSCs: bone marrow-derived mesenchymal stromal cells; ADMSCs: adipose-tissue-derived mesenchymal stromal cells; EPCs: endothelial progenitor cells; UMSCs: umbilical-cord-derived mesenchymal stromal cells.

patients who had no BM-MNC injection served as control subjects. The median follow-up period was 4.8 years. The 4-year amputation-free rates after BM-MNC implantation were 48% in PAD patients and 95% in Buerger's disease, while they were 0% in control PAD patients and 6% in control Buerger's disease. The 4-year overall survival rates after BM-MNC implantation were 76% in PAD patients and 100% in Buerger's disease, while they were 67% in control PAD patients and 100% in control Buerger's disease. Multivariable Cox proportional hazards analysis revealed that BM-MNC implantation correlated with prevention of major amputation and that hemodialysis and diabetes mellitus correlated with major amputation. In the patients with Buerger's disease, ABPI and TcPO2 were significantly increased after 1 month and remained high during the 3-year follow-up period. However, in patients with PAD, ABPI and TcPO2 significantly increased after 1 month but then gradually decreased during the 3-year follow-up period and returned to baseline levels. The authors concluded that BM-MNC transplantation is safe and effective in patients with CLI, especially in patients with Buerger's disease [27].

We have earlier evaluated and reported the results of a study of 13 patients in a phase I trial to investigate the safety and efficacy of intra-arterial injection of BM-MSCs in CLI patients. There was significant pain relief in these patients at 6 months after cell administration compared with their baseline level. Visual analog scale scores decreased from $2.29 \pm 0.29$ to $0.5 \pm 0.34$ ($P < 0.05$), ABPI increased significantly from $0.56 \pm 0.02$ to $0.67 \pm 0.021$ ($P < 0.01$), and TcPO2 also increased significantly in the foot from $13.57 \pm 3.63$ to $38 \pm 3.47$ ($P < 0.05$). Similar improvements were seen in the leg as well as in the thigh region. There was 86% limb salvage and six of seven ulcers showed complete or partial healing. The authors concluded that intra-arterial MSCs could be safely administered to patients with CLI and that significant therapeutic benefits were observed in these patients [46]. (A visual analog scale is a psychometric response scale that can be used in questionnaires. It is a measurement instrument for subjective characteristics or attitudes that cannot be directly measured. When responding to a visual analog scale item, respondents specify their level of agreement to a statement by indicating a position along a continuous line between two endpoints.)

The ulcer healing properties of BM-MSCs was demonstrated by Dash *et al.* [47]. This study was undertaken to assess the efficacy and feasibility of autologous BM-MSCs in the treatment of chronic nonhealing ulcers in patients with diabetic foot ulcers or Buerger's disease of the lower extremities. A total of 24 patients with nonhealing ulcers of the lower limb were enrolled and randomized into cell and control groups. In the cell group the patients received autologous cultured BM-MSCs together with standard wound dressing. The control group received only the standard wound dressing regimen. Patients were followed up for at least a 12-week period. Wound size, pain-free walking distance, and biochemical parameters were measured before therapy and at 2-week intervals following the intervention. The cell group had a significant improvement in pain-free walking distance and a reduction in ulcer size compared with those in the control group. There was no significant alteration in biochemical parameters observed during the follow-up period, indicating normal liver and renal function following the intervention. The study indicated that autologous implantation of BM-derived MSCs in nonhealing ulcers accelerated the healing process and significantly improved clinical parameters.

Lu *et al.* conducted a study to compare autologous bone-marrow-derived mesenchymal stem/stromal cells with the BM-MNCs for the treatment of diabetic CLI and foot ulceration [48]. The ulcer healing rate in the BM-MSC group was significantly higher than that in the BM-MNC group at 6 weeks after injection and reached 100% 4 weeks earlier than in the BM-MNC group. After 24 weeks of follow-up the improvements in limb perfusion induced by BM-MSC administration were more significant than those receiving BM-MNCs in terms of painless walking time, ABPI, TcPO2, and magnetic resonance angiography analysis. There was no significant difference between the groups in terms of pain relief and amputation and there were no serious adverse events related to either type of cell injections. The authors concluded that BM-MSC administration may be better tolerated and more effective than BM-MNCs for increasing lower limb perfusion and promoting foot ulcer healing in diabetic patients with CLI.

Teraa *et al.* conducted a meta-analysis of all randomized controlled trials (RCTs) that studied bone-marrow-derived cell therapy compared with standard care with or without placebo in CLI patients. Major amputation and amputation-free survival were considered as the primary endpoints. A total of 12 RCTs cumulatively including 510 CLI patients were analyzed. The meta-analysis showed significant beneficial effects of bone-marrow-derived cell therapy on both subjective and surrogate objective endpoints, including pain score, pain-free walking distance, ABPI, and TcPO2 measurements. Overall, the RCTs showed reduced amputation rates in the therapeutic arms of the trials with a relative risk (RR) of major amputation of 0.58 (95% confidence interval (CI), 0.40–0.84;$P = 0.004$). However, when only the placebo-controlled RCTs were considered, the beneficial effect on major amputation rates was considerably reduced and no longer significantly different (RR = 0.78; 95% CI, 0.40–1.51; $P = 0.46$). Amputation-free survival did not significantly differ between the bone-marrow-cell-treated group and the control group. This meta-analysis underlined the promising potential of bone-marrow-derived cell therapy in CLI patients, but importantly indicated the need for placebo-controlled clinical trials once safety and feasibility issues were satisfactorily resolved in phase I trials [49].

### 46.5.3 Clinical trials in India

Few clinical trials using allogeneic or autologous MSCs or MNCs in CLI have been conducted in India. The first initiative was undertaken by the Department of Biotechnology, Government of India, which conducted a multicenter trial using autologous BM-MNCs in patients with CLI, the results of which are yet to be published.

Motukuru *et al.* enrolled 36 patients with nonreconstructible Buerger's disease and injected autologous BM-MNCs into the calf muscles of the affected limbs in a clinical trial conducted at Jain Institute of Vascular Sciences, Bhagwan Mahaveer Jain Hospital, Karnataka, India [48]. They reported no procedure-related complications. Three patients (12%) underwent major amputations within 6 months. The others had improvement in ulcer healing, an increase in the mean ABPI, and an improvement in mean TcPO2 levels, resulting in limb salvage in all at 6 months. They concluded that BM-MNCs transplantation into ischemic limbs was a relatively safe procedure with no demonstrable side-effects at 6 months follow-up.

### 46.5.4 Stempeutics research experience in critical limb ischemia

The off-the-shelf allogeneic BM-MSC product developed by Stempeutics has undergone two clinical trials in CLI. The phase I/II trial included all patients with CLI (both atherosclerotic CLI and Buerger's disease), while in the phase II trial we exclusively included patients with CLI due to Buerger's disease as per Shionoya's diagnostic criteria [51]. These criteria are a smoking history, onset before the age of 50 years, infrapopliteal arterial occlusions, either upper limb involvement or phlebitis migrans, and absence of atherosclerotic risk factors other than smoking. All five criteria need to be present in order to make a diagnosis of Buerger's disease. Phlebitis is inflammation of a vein. When it occurs repeatedly in different locations it is known as thrombophlebitis migrans or migrating thrombophlebitis and can be an early indicator of gastric or pancreatic cancer.

### 46.5.5 Phase I/II study in patients with critical limb ischemia

The phase I/II results of stempeucel in CLI patients have been published [52]. Briefly, 10 patients each were randomized to either the cell arm or the placebo arm. A BM-MSC dose of 2 million MSCs/kg body weight administered intramuscularly was shown to be safe in patients with CLI. Improvement was observed in the rest pain scores in both arms, and no measurable differences were observed between the two arms. A significant

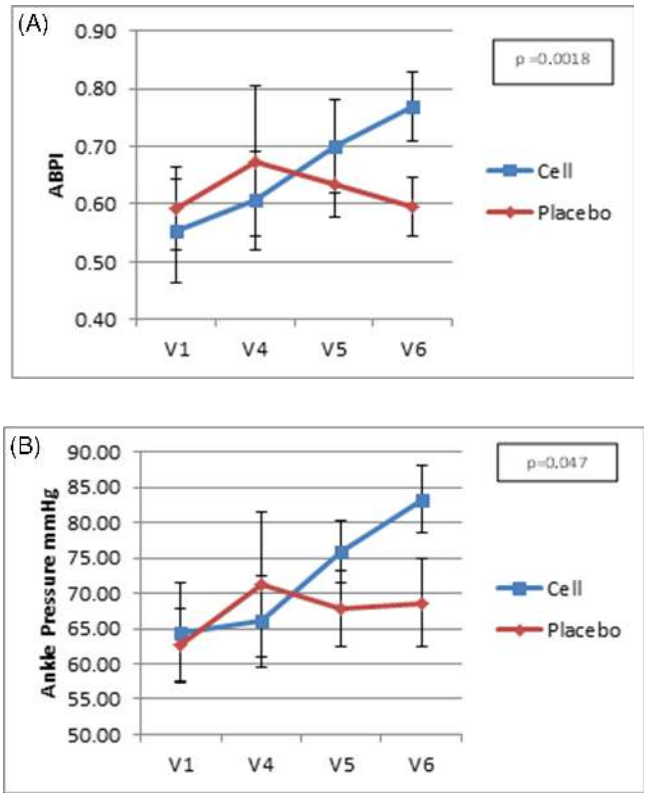



**Figure 46.7** Phase I/II study efficacy parameters. Results of the ankle-brachial index test (A) and ankle pressure (B) are shown as mean plus/minus standard deviation. VI: screening; V4: 1 month; V5: 3 months; V6: 6 months of follow-up.

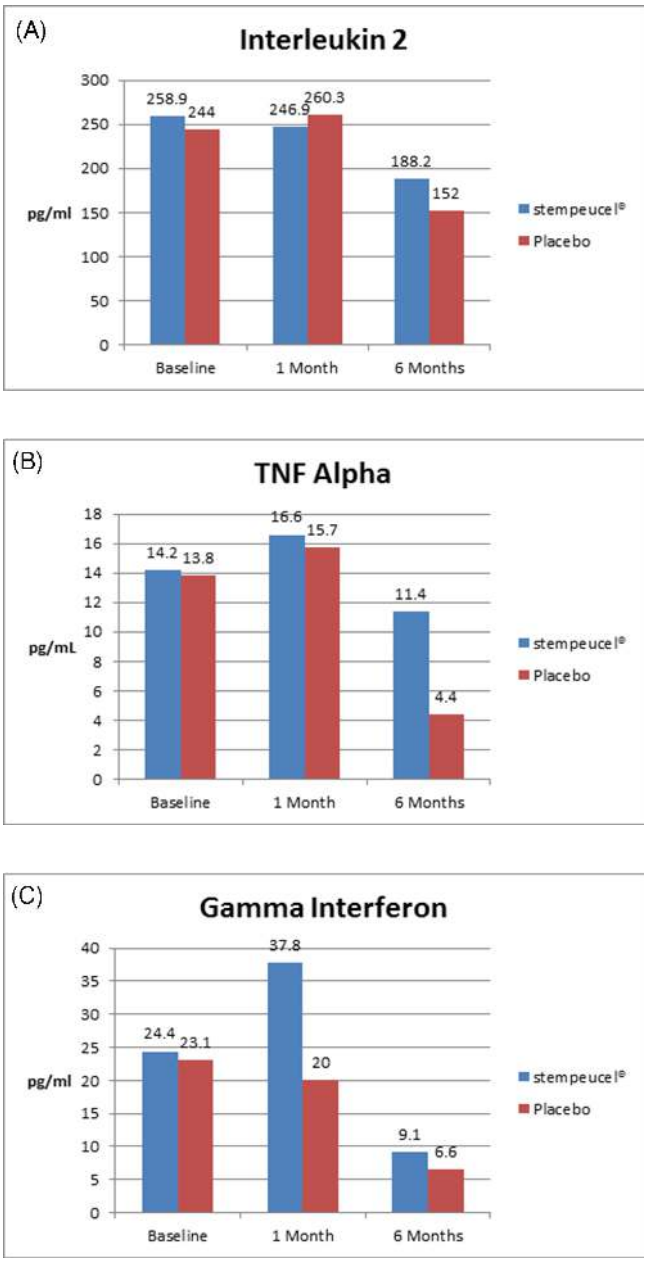



**Figure 46.8** Immunological response markers in stempeucel-treated arm and placebo arm at baseline, 1 month, and 6 months follow-up for IL-2 (A), TNF-α (B), and interferon-γ (C).

increase in ABPI and ankle pressure was seen in the MSC arm compared with the placebo group (Figure 46.7).

It is noteworthy that although a small group of 10 patients was recruited in each arm of the trial, we found a significant increase in the blood flow of patients in the lower limbs that received stempeucel. The incidence of adverse events in the BM-MSC arm was 13 versus 45 in the placebo arm, while the incidence of serious adverse events was similar in both the arms: five in the MSC group and four in the placebo group. The serious adverse events were related to disease progression and not related to the administration of cells or placebo.

In addition to routine safety laboratory parameters, such as measurement of parameters of renal and hepatic function, levels of the proinflammatory cytokines IL-2, TNF-α, and interferon-γ were documented. $CD4^+$, $CD8^+$, and $CD25^+$ lymphocyte numbers before and after stempeucel or placebo injection were measured by flow cytometry at baseline, 1 month, 3 months, and 6 months. Results showed that both immunological response (Figure 46.8) and CD markers (Figure 46.9) were comparable in both the placebo and cell arms.

No significant difference in blood lymphocyte profile or in serum cytokine level was observed between stempeucel and placebo-administered patients, suggesting that the administered allogeneic cells did not elicit a T cell proliferative response *in vivo*. With respect to the levels of the proinflammatory cytokines, we observed a possible difference between the MSC recipients and the placebo control patients at baseline, 1 month, and 6 months after treatment, but they were either comparable to the baseline values or were within the normal range of these cytokines. Collectively, these data indicate that allogeneic BM-MSC

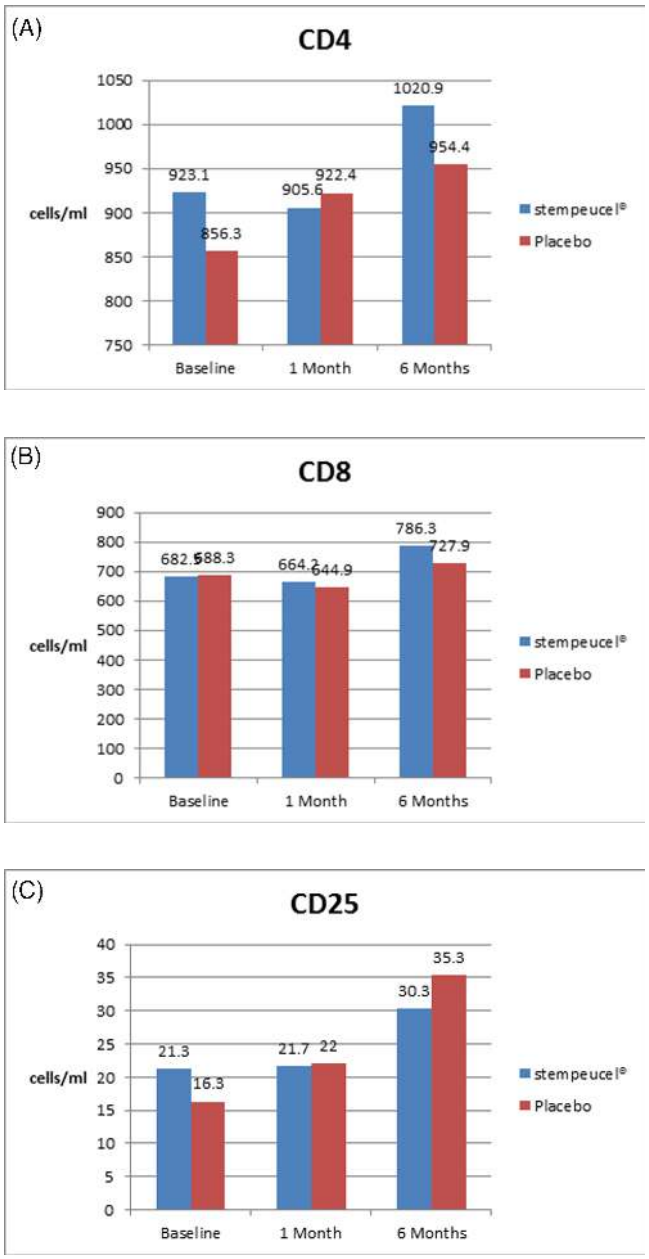



**Figure 46.9** CD markers in stempeucel arm and placebo arms at baseline, 1 month, and 6 months follow-up for $CD4^+$ cells (A), $CD8^+$ cells (B) and $CD25^+$ cells (C).

administration in CLI patients did not adversely alter the immunological profile.

It was concluded that BM-MSCs were safe when injected intramuscularly at a dose of 2 million cells/kg body weight. The safety profile exhibited by BM-MSCs, coupled with the observed improvements in some of the important clinical parameters in Buerger's disease, warranted a separate study with a larger number of patients. An open-label nonrandomized dose-finding phase II study was next conducted in patients with Buerger's disease.

### 46.5.6 Phase II study in patients with Buerger's disease

The second trial initiated in patients with CLI due to Buerger's disease was an open-label, nonrandomized, dose-finding phase II study evaluating different dose levels of stempeucel in patients with CLI due to Buerger's disease. Thirty-six patients were accrued to one arm of the study and received 1 million MSCs/kg, and 36 were accrued to the second arm of the study and received 2 million MSCs/kg. Eighteen patients were accrued to the control arm. Patients in the cell groups received the respective dose of stempeucel into the calf muscle of the affected limb and 2 mL of stempeucel around ischemic ulcers. Patients belonging to the control arm received standard of care only. Six months follow-up of the patients has been completed, and these patients will be followed up for 2 years.

The safety profiles of both the 1 million MSCs/kg and 2 million MSCs/kg groups were comparable to that of control group. All the adverse events were manifestations of the underlying disease. None of the serious adverse events reported were related to stempeucel as per the investigators' feedback and were due to progression of the disease. There was significant improvement in both the primary endpoints of the trial: relief of rest pain (Figure 46.10) and healing of ulceration.

Furthermore, stempeucel recipients showed a significant increase in blood flow in the lower limbs, as evidenced by a significant increase in ankle systolic pressure (Figure 46.11) and total walking distance in the 2 million MSCs/kg dose group.

Hence, it was concluded that BM-MSCs at a dose of 2 million MSCs/kg body weight has a role in alleviating the symptoms of CLI in Buerger's disease.

## 46.6 Conclusions

CLI represents the most severe manifestation of PAD, diminishes quality of life and global function, and is often associated with very high short-term mortality. Prompt clinical diagnosis, vasodilator agents, and surgical revascularization represent the current standard of care. However, in some patients this care is either not possible or ineffective in providing clinical benefit. Effective new biological and angiogenic therapies would fill a real clinical need. Current literature favors the administration of autologous BM-MNCs or allogeneic BM-MSCs; the latter are relatively safe, easy to expand *ex vivo*, and appear to be efficacious. Issues that remain unresolved include the optimal cell type, optimum cell dosage, and optimal route of administration. Cells that have immunomodulatory and proangiogenic properties appear likely to be the cell of choice for the treatment of CLI due to PAD.

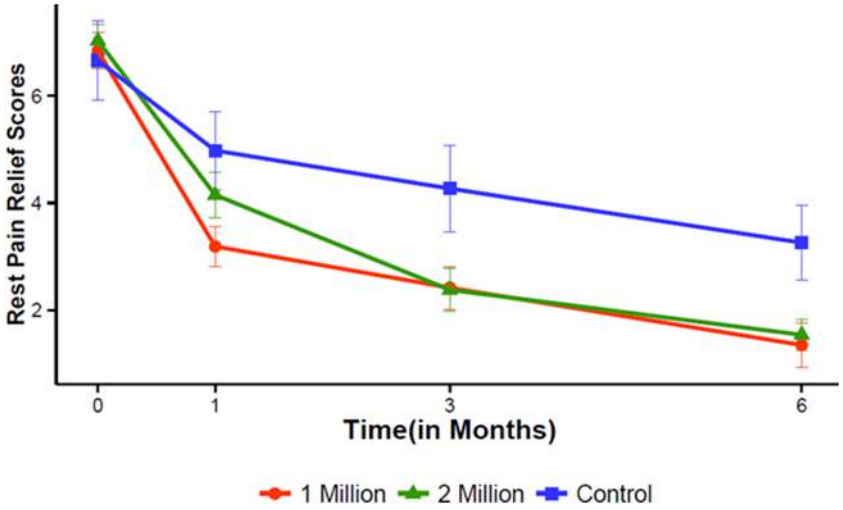



**Figure 46.10** Phase II study results of rest pain scores, shown as mean plus/minus standard error, at time of screening and at 1 month, 3 months, and 6 months follow-up.

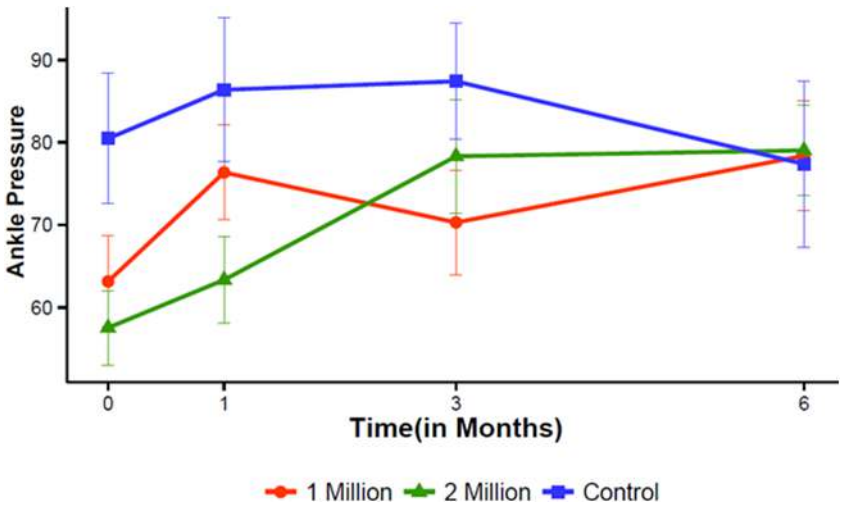



**Figure 46.11** Phase II study results of ankle pressure index, shown as mean plus/minus standard error, at time of screening, and at 1 month, 3 months, and 6 months follow-up.

## References


1 A. T. Hirsch, Z. J. Haskal, N. R. Hertzer, *et al.* ACC/AHA 2005 Practice guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic):a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* 2006;**113**(11): e463–e654.

2 G. Piazza and M. A. Creager. Thromboangiitis obliterans. *Circulation* 2010;**121**(16):1858–1861.

3 J. W. Olin. Thromboangiitis obliterans (Buerger's disease). *N. Engl. J. Med.* 2000;**343**(12):864–869.

4 J. J. Belch, E. J. Topol, G. Agnelli, *et al.* Critical issues in peripheral arterial disease detection and management: a call to action. *Arch. Intern. Med.* 2003;**163**(8):884–892.

5 M. H. Criqui. Peripheral arterial disease – epidemiological aspects. *Vasc. Med. Lond. Engl.* 2001;**6**(3 Suppl):3–7.

6 W. R. Hiatt, A. T. Hirsch, J. G. Regensteiner, and E. P. Brass. Clinical trials for claudication assessment of exercise performance, functional status, and clinical end points. *Circulation* 1995;**92**(3):614–621.

7 W. S. Aronow. Management of peripheral arterial disease of the lower extremities in elderly patients. *J. Gerontol. A. Biol. Sci. Med. Sci.* 2004;**59**(2):M172–M177.

8 J. L. Mills and J. M. Porter. Buerger's disease (thromboangiitis obliterans). *Ann. Vasc. Surg.* 1991;**5**(6):570–572.

9 B. Sigvant, K. Wiberg-Hedman, D. Bergqvist, *et al.* A population-based study of peripheral arterial disease prevalence with special focus on critical limb ischemia and sex differences. *J. Vasc. Surg.* 2007;**45**(6):1185–1191.

10 R. W. Sprengers, D. J. Lips, F. L. Moll, and M. C. Verhaar. Progenitor cell therapy in patients with critical limb ischemia without surgical options. *Ann. Surg.* 2008;**247**(3):411–420.
11 H. Lawall, P. Bramlage, and B. Amann. Treatment of peripheral arterial disease using stem and progenitor cell therapy. *J. Vasc. Surg.* 2011;**53**(2):445–453.
12 The European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Human Use. Note for guidance on clinical investigation of medicinal products for the treatment of peripheral arterial occlusive disease. CPMP/EWP/714/98 rev 1, April 25, 2002.
13 A. J. Friedenstein, J. F. Gorskaja, and N. N. Kulagina. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 1976;**4**(5):267–274.
14 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.
15 D. J. Prockop. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;**276**(5309):71–74.
16 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411):143–147.
17 S. Aggarwal and M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.
18 J. Stagg. Immune regulation by mesenchymal stem cells: two sides to the coin. *Tissue Antigens* 2007;**69**(1):1–9.
19 A. I. Caplan. What's in a name? *Tissue Eng. Part A* 2010;**16**(8):2415–2417.
20 A. I. Caplan and D. Correa. The MSC: an injury drugstore. *Cell Stem Cell* 2011;**9**(1):11–15.
21 T. J. Burdon, A. Paul, N. Noiseux, *et al.* Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. *Bone Marrow Res.* 2011;**2011**:e207326.
22 M. B. Murphy, K. Moncivais, and A. I. Caplan. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp. Mol. Med.* 2013;**45**:e54.
23 L. Chen, E. E. Tredget, P. Y. G. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3**(4):e1886.
24 A. M. Hocking and N. S. Gibran. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp. Cell Res.* 2010;**316**(14):2213–2219.
25 C. Botti, C. Maione, A. Coppola, *et al.* Autologous bone marrow cell therapy for peripheral arterial disease. *Stem Cells Cloning* 2012;**5**:5–14.
26 A. I. Caplan. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol.* 2007;**213**(2):341–347.
27 N. Idei, J. Soga, T. Hata, *et al.* Autologous bone-marrow mononuclear cell implantation reduces long-term major amputation risk in patients with critical limb ischemia: a comparison of atherosclerotic peripheral arterial disease and Buerger disease. *Circ. Cardiovasc. Interv.* 2011;**4**(1):15–25.
28 N. Lehman, R. Cutrone, A. Raber, *et al.* Development of a surrogate angiogenic potency assay for clinical-grade stem cell production. *Cytotherapy* 2012;**14**(8):994–1004.
29 European Medicines Agency, Evaluation of Medicines for Human Use. Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer. EMEA/CHMP/BWP/271475/2006, October 10 2007.
30 C. A. Bravery, J. Carmen, T. Fong, *et al.* Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy* 2013;**15**(1):9–19.
31 K. J. Ledford, F. Zeigler, and R. L. Bartel. Ixmyelocel-T, an expanded multicellular therapy, contains a unique population of M2-like macrophages. *Stem Cell Res. Ther.* 2013;**4**(6):134.
32 R. Pal, M. Hanwate, M. Jan, and S. Totey. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J. Tissue Eng. Regen. Med.* 2009;**3**(3):163–174.
33 M. K. Mamidi, K. G. Nathan, G. Singh, *et al.* Comparative cellular and molecular analyses of pooled bone marrow multipotent mesenchymal stromal cells during continuous passaging and after successive cryopreservation. *J. Cell. Biochem.* 2012;**113**(10):3153–3164.
34 S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 2012;**10**(3):244–258.
35 T. Kinnaird, E. Stabile, M. S. Burnett, *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ. Res.* 2004;**94**(5):678–685.
36 N. Ferrara and T. Davis-Smyth. The biology of vascular endothelial growth factor. *Endocr. Rev.* 1997;**18**(1):4–25.
37 T. Pritchett and L. Little. "Hard cell": potency testing for cellular therapy products. *Bioprocess. Int.* 2012;**10**:36–38.
38 J. C. Ra, I. S. Shin, S. H. Kim, *et al.* Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev.* 2011;**20**(8):1297–1308.
39 I. A. Isakova, K. Baker, J. Dufour, *et al.* Preclinical evaluation of adult stem cell engraftment and toxicity in the CNS of rhesus macaques. *Mol. Ther.* 2006;**13**(6):1173–1184.
40 D. J. Prockop and S. D. Olson. Clinical trials with adult stem/progenitor cells for tissue repair: let's not overlook some essential precautions. *Blood* 2007;**109**(8):3147–3151.
41 E. Eggenhofer, V. Benseler, A. Kroemer, *et al.* Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Alloimmunity Transplant.* 2012;**3**:297.
42 Y. Kim, H. Kim, H. Cho, *et al.* Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. *Cell. Physiol. Biochem.* 2007;**20**(6):867–876.
43 T. Iwase, N. Nagaya, T. Fujii, *et al.* Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc. Res.* 2005;**66**(3):543–551.
44 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.
45 E. Tateishi-Yuyama, H. Matsubara, T. Murohara, *et al.* Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002;**360**(9331):427–435.
46 A. K. Das, B. J. J. Bin Abdullah, S. S. Dhillon, *et al.* Intra-arterial allogeneic mesenchymal stem cells for critical limb ischemia are safe

and efficacious: report of a phase I study. *World J. Surg.* 2013;**37**(4):915–922.

47 N. R. Dash, S. N. Dash, P. Routray, *et al.* Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuvenation Res.* 2009;**12**(5):359–366.

48 D. Lu, B. Chen, Z. Liang, *et al.* Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res. Clin. Pract.* 2011;**92**(1):26–36.

49 M. Teraa, R. W. Sprengers, Y. van der Graaf, *et al.* Autologous bone marrow-derived cell therapy in patients with critical limb ischemia: a meta-analysis of randomized controlled clinical trials. *Ann. Surg.* 2013;**258**(6):922–929.

50 V. Motukuru, K. R. Suresh, V. Vivekanand, *et al.* Therapeutic angiogenesis in Buerger's disease (thromboangiitis obliterans) patients with critical limb ischemia by autologous transplantation of bone marrow mononuclear cells. *J. Vasc. Surg.* 2008;**48**(6 Suppl):53S–60S, discussion 60S.

51 S. Shionoya. Diagnostic criteria of Buerger's disease. *Int. J. Cardiol.* 1998;**66**(Suppl 1):S243–S245, discussion S247.

52 P. K. Gupta, A. Chullikana, R. Parakh, *et al.* A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J. Transl. Med.* 2013;**11**:143.

CHAPTER 47

# The role of mesenchymal stromal cells in the management of musculoskeletal disorders

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## 47.1 Summary

Mesenchymal stromal cells (MSCs) are a population of progenitor cells that can be easily isolated from bone marrow, adipose tissue, and other sources and culture expanded for cell-based therapeutic applications. MSCs can be differentiated into various mesenchymal tissue types, including bone, cartilage, muscle, and tendon, and thus play a pivotal role in the regeneration of musculoskeletal tissues. This chapter describes the potential of MSCs for site-specific and systemic tissue regeneration applications and summarizes the current preclinical and clinical evidence.

## 47.2 Introduction

Cells are key players in the treatment of musculoskeletal disorders. Interest in basic and clinical research of MSCs has increased dramatically during the last two decades. MSCs have the potential to differentiate into various types of cells of mesenchymal lineage. They have provided hope for regenerative medicine in the future. The International Society for Cellular Therapy proposed minimal criteria to define human MSCs in 2008 [1]. First, MSCs are plastic-adherent when maintained in standard culture conditions. Second, MSCs express CD105, CD73, and CD90, and lack expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR surface molecules. Third, MSCs are able to differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro*.

MSCs were first isolated from murine bone marrow by Friedenstein *et al.* [2]. Since then they have been isolated from almost tissues, including adipose tissue [3], trabecular bone [4], periosteum [5], synovial membrane [6], skeletal muscle [7], dental pulp [8], placenta, and others [9]. However, the most commonly used sources for MSCs are bone marrow (Figure 47.1) and adipose tissue [11]. Tissue-specific stem-like cells and precursor cells from the bone marrow collaborate for tissue repair [12].

Currently, two strategies exist for preparing MSCs for clinical applications (Figure 47.2), each with its advantages and disadvantages.

Applying *instant* cell therapy, bone marrow aspirates containing approximately $1 \times 10^2$ MSCs/mL are harvested, usually from the iliac crest [13]. MSCs are then concentrated by centrifugation

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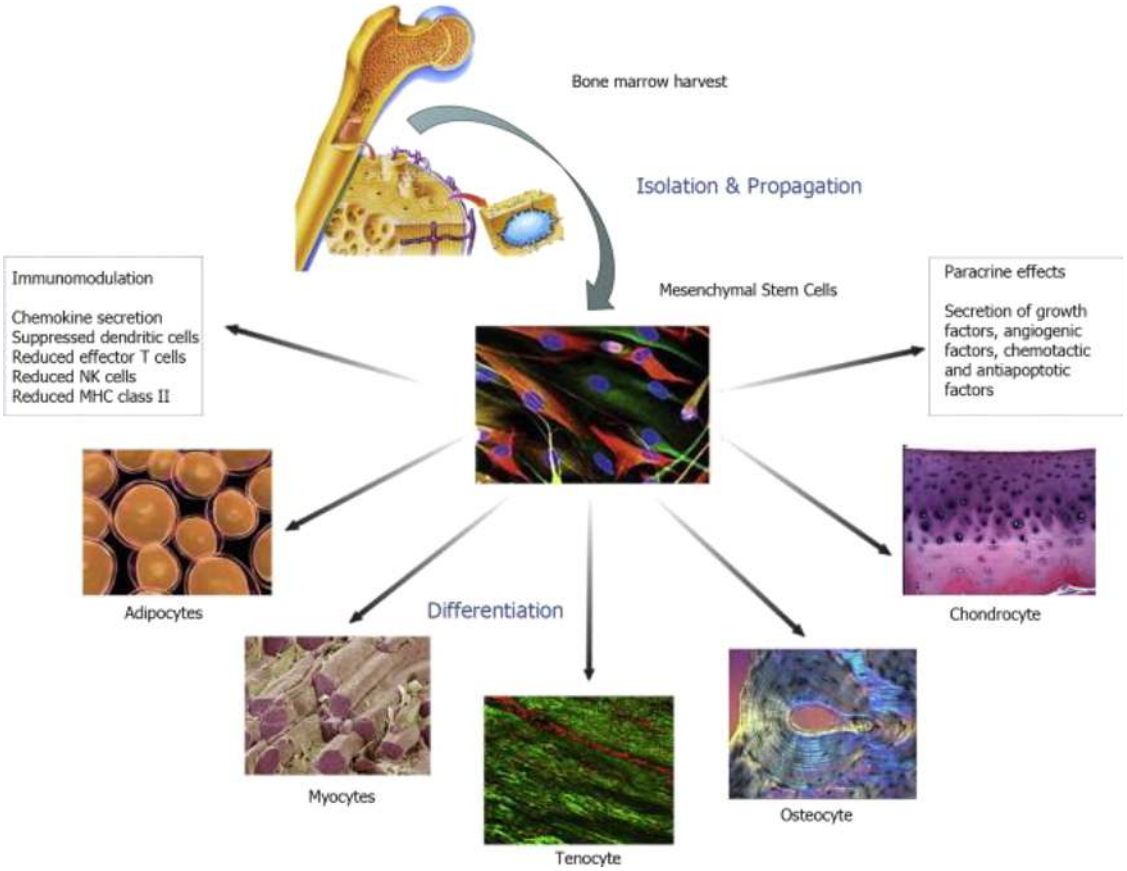



**Figure 47.1** MSCs can be isolated from numerous connective (and other) tissues, but bone marrow has been the most widely used source to date. The effects of MSCs may be mediated by differentiation toward the target cell lineage (at least in the case of the musculoskeletal system) and by their secretion of trophic (supportive) factors via paracrine and autocrine activity, including angiogenic, chemoattractant, antiapoptotic, and anti-inflammatory factors, as well as by immunomodulation of T cells, B cells, and natural killer cells. (See color plate section.) *Source:* Nixon [10], pp. 278–294. Reproduced with permission of Elsevier.

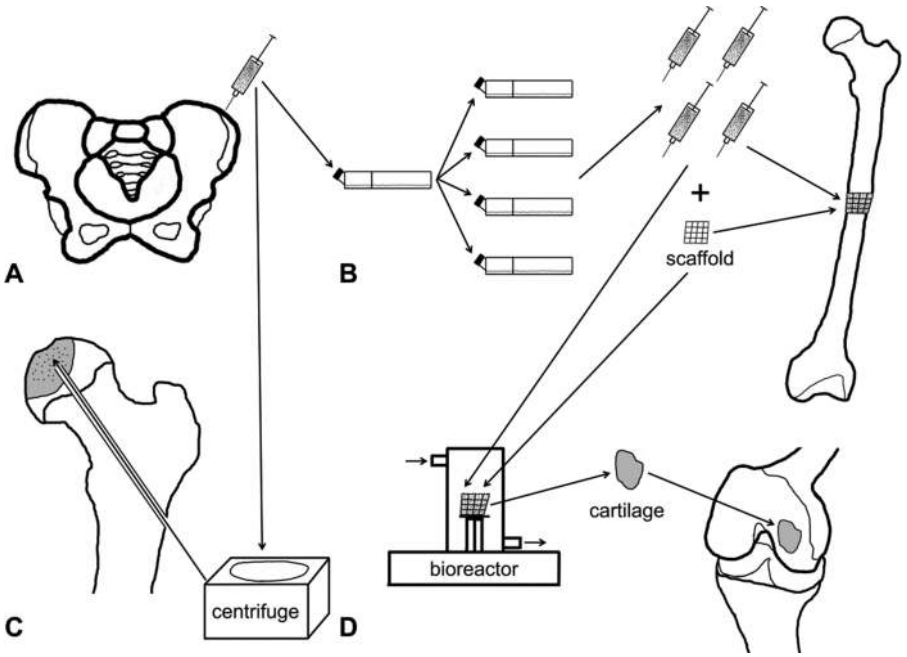



**Figure 47.2** Options for using MSCs in the treatment of musculoskeletal disorders. (A) Bone marrow is most commonly aspirated from the iliac crest. Thereafter, there are various ways to process the cells for their administration to the patient. For "immediate" administration the bone marrow aspirate is concentrated by centrifugation and placed directly at the site of injury. As an example, this is used clinically to treat early stages of osteonecrosis of the femoral head. (C) Cells concentrated by centrifugation are applied after core decompression. Alternatively, to generate large numbers of cells, MSCs are isolated from the bone marrow aspirate and expanded in *ex vivo* culture for several weeks (B). A large number of cells can be transplanted, often combined with a scaffold. A critical-size femoral bone defect would be approached in this way. Finally, MSCs can be isolated, expanded, and differentiated in culture. An example is the production of cartilage by culturing MSCs in chondrogenic media in a bioreactor (D).

by a factor of about 14 and directly administered to the site of injury [13]. The major advantage of this method is that only one operation is needed to harvest the cells and use them for treatment. The disadvantage is that only comparatively low absolute cell numbers can be generated by this process.

Alternatively, MSCs can be isolated and expanded *in vitro* before transplantation. Large numbers of cells can be generated. MSCs can be isolated from bone marrow by various methods, including the use of immunomagnetic beads, density gradient separation, and direct plating [14]. Polystyrene tissue culture flasks or factories are subsequently used for *ex vivo* expansion of the MSCs. After a certain number of passages MSCs start to lose their multilineage mesenchymal differentiation qualities [15]. First, their osteogenic and adipogenic, and later their chondrogenic, potentials are affected [16]. In contrast to the small number of MSCs obtained from a marrow aspirate that is administered immediately, about $1.0–4.8 \times 10^9$ MSCs can be harvested from 12 mL bone marrow after cell culture to passage 3 [17]. The two-step method has two disadvantages: first, one additional interventional step including some type of regional anesthesia is required for implantation of the cells into a musculoskeletal site; and second, there is a risk of infection during *ex vivo* expansion.

In preclinical and clinical studies, MSCs can be delivered locally [18–21] or systemically [22,23].

A promising future tool for differentiation into cells of any type are induced pluripotent stem cells. In 2006, Yamanaka *et al.* showed that somatic cells such as fibroblasts could be reprogrammed to pluripotent cells by the transfer of stem cell genes [24]. Although a small number of clinical trials have been initiated with the differentiated progeny of both induced pluripotent stem cells and embryonic stem cells, the technology required is infinitely greater than that required for the use of MSCs.

## 47.3 Stem cells for bone regeneration

Osteoblasts produce osteoid, an extracellular protein-based matrix, which mineralizes to become bone. MSCs are osteoprogenitors that can differentiate into osteoblasts and induce bone formation.

### 47.3.1 Bone defects

Treating and healing bone diseases is often challenging. Critical-size bone defects (Figure 47.3) are defects of a size and location that do not heal by themselves. They can occur due to trauma, infection, failure of total joint replacement, or tumor or be associated with other musculoskeletal disorders. The gold standard for treatment of these defects is autologous bone grafting. However, this procedure has limited availability and donor site morbidity. Numerous preclinical studies have demonstrated the advantages of MSCs for bone regeneration [25–29]. Human clinical trials employing MSCs are examining their potential for bone regeneration applications [30,31].

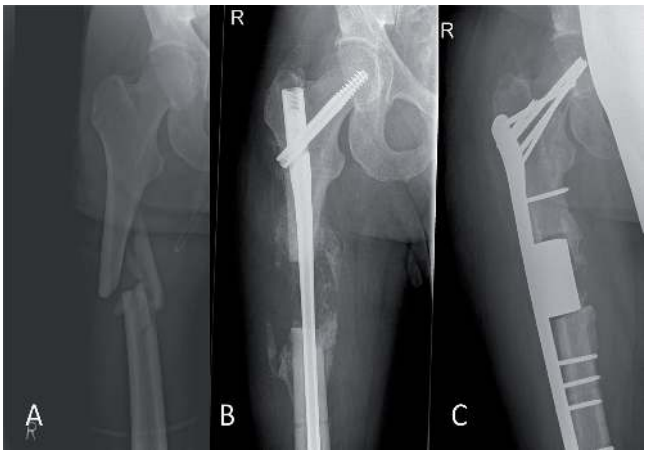



**Figure 47.3** (A) A critical-size femoral bone defect is shown. The 33-year-old male patient suffered a diaphyseal multifragmentary fracture of the femur. (B) After primary osteosynthesis failed, the two-stage Masquelet technique of membrane formation and bone grafting was applied. Unfortunately, the patient developed a deep wound infection requiring further revision surgery, including temporary external fixation and filling of the defect using antibiotic-releasing bone cement in addition to systemic antibiotic treatment. (C) In the next step a trabecular tantalum-coated void filler was implanted and stabilized by a locked plate. To achieve long-term regeneration of such extended bone defects, a strategy that includes an osteoconductive bioresorbable scaffold material in combination with osteoprogenitor cells (e.g., MSCs) and osteoinductive factors is needed.

Currently, various strategies are used to treat bone defects. The ideal bone graft material has osteoconductive, osteoinductive, and osteogenic properties [32].

For bone regeneration, MSCs provide osteoinductive and osteogenic properties because of their secretion of appropriate growth factors and cytokines and can differentiate into osteoblasts [32]. MSCs also have immune-modulating properties and can suppress excessive and damaging inflammation.

Treatment of critical-size bone defects with MSCs usually requires a biocompatible scaffold to support their growth and induce bone formation [33]. Bruder *et al.* isolated bone-marrow-derived MSCs, expanded them, and seeded them on to porous hydroxyapatite–tricalcium phosphate (TCP) ceramic scaffolds. The implants were transplanted into an 8 mm critical-size femoral bone defect in rats. After 4 weeks 2%, after 8 weeks 23%, and after 12 weeks 46% of the available void in the scaffolds was filled with bone [25]. Arinzeh *et al.* used expanded allogeneic MSCs loaded on to hollow TCP cylinders and transplanted this construct into a 21 mm (critical-size) femoral bone defect in dogs [26]. No adverse host response was detected after 16 weeks of observation. The MSC-loaded implants showed significantly more bone growth than the control implants without MSCs.

Total joint replacement is a common procedure for patients with severe osteoarthritis (OA) involving the hip. The majority of these patients have a successful outcome. However, for a number of reasons, including infection, aseptic loosening, osteolysis due to wear debris, and trauma, bone implants can become loose and result in major bone defects (Figure 47.4).

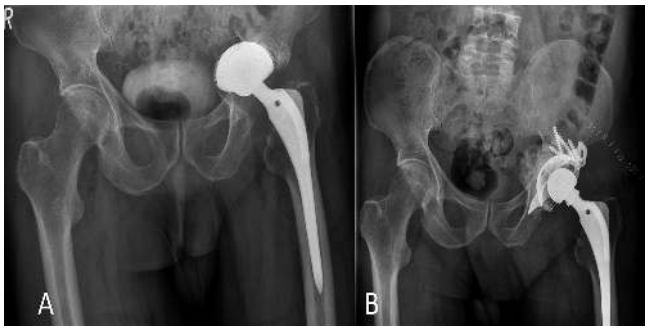



**Figure 47.4** (A) A large bony periprosthetic pelvic defect with acetabular implant loosening 2 years after total hip replacement for femoral neck fracture is shown. (B) Revision surgery was performed, implanting a cemented cup into an antiprotrusio ring supported with bone allograft. The use of one- or two-stage isolated autologous MSCs seeded on a bioresorbable scaffold material is a promising strategy to avoid the disadvantages associated with bone allografting, including reduced osteointegration of the implant and the risk for transmission of infectious agents.

Korda *et al.* investigated the impact of allografts enriched with MSCs in an ovine (sheep) hip hemiarthroplasty model [29]. Bone-marrow-derived MSCs were expanded and mixed with allograft bone prior to implantation into the defect. The distal part of the stem of the artificial joint was implanted with cement, whereas the proximal part was impaction-grafted by a mixture of allograft bone with or without MSCs. After 12 weeks there was significantly more bone formation surrounding the implants that were impaction-grafted with the mixture of MSCs and allograft bone compared with the group with bone allografts without MSCs.

Immediate cell therapy for the treatment of acetabular bone defects after failed total hip replacement was described by Ochs *et al.* [34]. Autologous bone marrow was used to enrich freeze-dried irradiated bone allografts for acetabular reconstruction. After an average of 31.4 months follow-up each hip showed evidence of trabeculation and incorporation of the allograft with no acetabular loosening.

Stromal cell-derived factor-1 plays a key role in the attraction of MSCs to sites of bony defects [35]. It also enhances the bone inductive capabilities of bone morphogenetic protein (BMP)-2 and thus reduces the necessary concentration of BMP-2. High BMP-2 concentrations can be associated with significant side-effects, such osteonecrosis [36].

Intervertebral disc degeneration with vertebral instability resulting in pain and sensorimotor deficits is one of the commonest causes of low back pain. Treatment options are limited to conservative treatment and surgical fusion to eliminate the instability. Preclinical studies and initial clinical trials have described the potential of MSCs to support disc regeneration or spinal fusion. Pang *et al.* expanded human umbilical-cord-tissue-derived MSCs to passages 6–8 and injected them locally into injured discs [37]. This resulted in improved function and reduced pain. Yang *et al.* developed a rabbit model for anterior spinal fusion using two biological matrix-assisted treatment groups of porous collagen sponge plus cultured and osteogenically differentiated MSCs versus porous collagen sponge alone. They compared bone regeneration with an autologous bone graft and no implant as positive and negative controls respectively [38]. The autologous bone graft resulted in a 70% fusion rate after 12 weeks, while the collagen sponge with MSCs had a 40% fusion rate. The collagen sponge alone and no implant control groups did not result in any bony fusion. The authors suggested that ongoing research on MSC differentiation and scaffold composition may further improve the outcome of MSC-seeded scaffolds for spinal fusion.

Despite promising animal studies and clinical trials, Khashan *et al.* concluded in a systematic review of the literature that there is not sufficient evidence to support the use of a bone marrow aspirate combined with an allograft, or synthetic scaffold as a substitute for iliac crest grafts or as a supplement for any autologous bone grafts, for thoracolumbar spinal fusion [39]. Weak evidence was found to support the use of bone marrow aspirates as a substitute for local bone grafts during thoracolumbar spinal fusion procedures [39].

### 47.3.2 Osteonecrosis

The pathophysiology of osteonecrosis (also known as avascular necrosis or aseptic necrosis) is controversial. Different theories include a disorder in lipid metabolism, abnormal clotting disorders, genetic abnormalities, or increased intraosseous pressure that leads to decreased blood flow and cell death [40]. Risk factors include the systemic use of corticosteroids, chemotherapy, alcoholism, trauma, decompression sickness, vascular compression, hypertension, vasculitis, arterial embolism and thrombosis, radiation, bisphosphonates (particularly affecting the mandible), sickle cell anemia, systemic lupus erythematosus, and Gaucher's disease. In some cases it is idiopathic (no cause found). Osteonecrosis results in severe pain and functional deficits, especially when the disease is located at the hip or knee. Early stages of the disease are only detectable by magnetic resonance imaging (MRI) or bone scan. Treatment with core decompression is recommended for stages prior to subchondral bone collapse. For example, core decompression for osteonecrosis of the femoral head involves drilling one larger hole or several smaller holes into the femoral head to relieve pressure in the bone and create channels for new blood vessels to supply the affected areas of the hip. If the subchondral bone collapse has already occurred, total joint replacement is the only long-term therapeutic option.

A combination of core decompression and concentrated bone marrow aspirate application was successful for cases up to Ficat and Arlet stage II osteonecrosis of the femoral head (Figure 47.5) [41–44]. The Ficat staging system ranges from stage 0 (in which the patient has no symptoms, a normal X-ray, and a normal magnetic resonance image) to stage IV (in which patients walk with a painful limp and both X-rays and MRI show secondary degenerative changes).

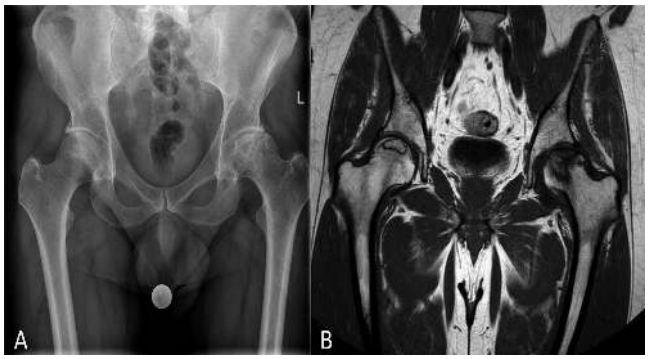



**Figure 47.5** (A) An anteroposterior X-ray of the pelvis and (B) a magnetic resonance image of a 36-year-old male patient with osteonecrosis of the femoral head. The right hip shows subchondral edema only detectable by MRI and corresponding to Association Research Circulation Osseous (ARCO) stage I. ARCO staging primarily uses X-ray and MRI to determine the stage of osteonecrosis. Core decompression combined with autologous bone marrow concentrate or expanded osteoprogenitor cells is a promising treatment option. (B) The left hip (L) shows stage IV osteonecrosis of the femoral head with complete destruction of the femoral head. Total joint replacement is the only current treatment to improve function and pain of the collapsed femoral head.

Ma *et al.* performed a randomized double-blinded study of 53 people with avascular necrosis of the femoral head [42]. Patients in the treatment group received core decompression combined with implantation of concentrated autologous bone marrow cells. The control group received only the core decompression. After 2 years the nonprogression rate for those with stage I/II disease was 100% in the treatment group compared with 66.7% in the control group. Hernigou *et al.* treated 38 patients – who had a total of 56 osteonecrotic hips associated with sickle cell disease – with core decompression and implantation of autologous bone marrow concentrate [45]. They found that autologous bone marrow grafting was a safe and effective treatment for early stage disease related to sickle cell disease compared with other studies describing the natural history of osteonecrosis of the femoral head.

### 47.3.3 Wear-particle-related osteolysis

The term osteolysis generally refers to a problem common to artificial joint replacements, such as total hip replacements, total knee replacements, and total shoulder replacements. Osteolysis can also be associated with the radiographic changes seen in a person with bisphosphonate-related osteonecrosis of the jaw. There are several biological mechanisms that may lead to osteolysis. In total hip replacement, the generally accepted explanation for osteolysis involves wear particles (worn off the contact surface of the artificial ball and socket joint). As the body attempts to clean up these wear particles (typically consisting of plastic or metal) it triggers an autoimmune reaction that causes resorption of bone tissue.

Total hip replacement achieves excellent long-term results and cost-effective improvement in pain and functional outcome [46]. Depending on the materials used for the bearing couple, metal, ceramic, conventional polyethylene, or highly crosslinked polyethylene particles are generated. Gibon *et al.* reported that chemokine C–C motif receptor 1 mediates the systemic recruitment of MSCs to ultra-high molecular weight polyethylene particles and recruited MSCs that were able to differentiate into functional osteoblasts and decrease particle-associated bone loss [47].

### 47.3.4 Systemic bone diseases

#### 47.3.4.1 Osteoporosis

Osteoporosis is a disorder of excessive skeletal fragility that results from a combination of a reduction in bone mass and bone strength. Its severity is measured by bone mineral density testing. This can be assessed in a number of ways, of which dual-energy X-ray absorptiometry is one of the most commonly used. The two most significant determinants of osteoporosis are estrogen deficiency and aging [48]. Zhang *et al.* compared the effect of systemic and local injection of gene-modified BMP-4-overexpressing MSCs on bone mineral density in osteoporotic mice [49]. Twenty-four hours after systemic administration only 0.02–0.29% of the cells were present in the femoral bone marrow. In contrast, 7 to 100 times more MSCs were present when the cells were injected locally. While bone volume and bone mineral density after systemic therapy remained unchanged, local injection of MSCs increased bone mineral density by 10.4% after 56 days [49]. MSCs injected systemically were found in the upper metaphysis, epiphysis, bone marrow sinusoids, or the osteon/Haversian systems and were usually removed from the bone marrow within a few weeks [48]. The osteon or Haversian system is the fundamental functional unit of much compact bone. Osteons are roughly cylindrical structures that are typically several millimeters long and around 0.2 mm in diameter. Each osteon consists of concentric layers, or lamellae, of compact bone tissue that surround a central canal, the Haversian canal. The Haversian canal contains the bone's nerve and blood supplies. The boundary of an osteon is the cement line (Figure 47.6).

Yao and Lane described a method of directing MSCs to the osteoporotic bone of both young and aged mice [48]. The synthetic high affinity and specific peptidomimetic ligand LLP2A, which targets integrin 1, a molecule highly expressed on MSCs, was linked to a bisphosphonate. Mice treated with the combination of LLP2A linked to the bisphosphonate and MSCs resulted in significant gains in both trabecular and cortical bone mass and strength.

#### 47.3.4.2 Osteogenesis imperfecta

Osteogenesis imperfecta is a congenital bone disorder characterized by brittle bones that are prone to fracture (Figure 47.7). Children with osteogenesis imperfecta are born with defective connective tissue, usually because of a deficiency of type-I collagen [50].

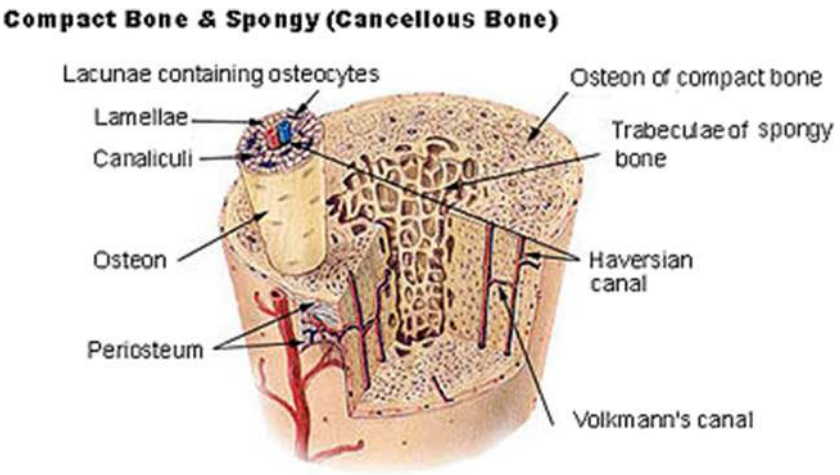



**Figure 47.6** Diagram of a typical long bone showing both compact (cortical) and cancellous (spongy) bone. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain. Its attribution is as follows: "Illu compact spongy bone" by SEER – US National Cancer Institute's Surveillance, Epidemiology and End Results (SEER) Program (http://training.seer.cancer.gov/anatomy/skeletal/tissue.html). Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Illu_compact_spongy_bone.jpg#/media/File:Illu_compact_spongy_bone.jpg.

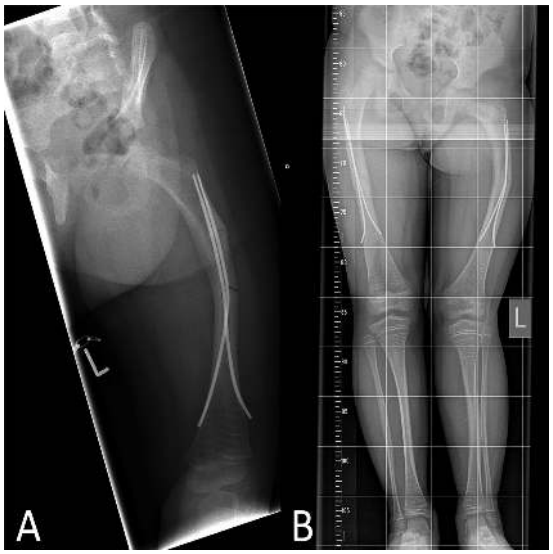



**Figure 47.7** Anteroposterior X-rays of a 5-year-old child with osteogenesis imperfecta. Two diaphyseal fractures were stabilized by intramedullary nails after closed reduction. Five years later multiple fractures had occurred. Stem/progenitor cell administration, either as an intravenous infusion of allogeneic MSCs or allogeneic bone marrow, has the potential to improve bone metabolism in this disease.

Horwitz *et al.* showed that allogeneic bone marrow transplantation in children with osteogenesis imperfecta led to donor-derived osteopoiesis and consequent improvement in the microscopic structure of bone [22]. Although MSC transplantation is a successful treatment of osteogenesis imperfecta, MSCs do not substantially engraft in bone; rather, they secrete a soluble mediator that indirectly stimulates bone growth [51].

## 47.4 Stem cells for cartilage regeneration

Articular cartilage is a simple avascular, aneural connective tissue that covers the ends of the articulating surfaces of bones [52,53]. It facilitates smooth joint movement in addition to dissipating biomechanical loads of the diarthrodial (synovial) joints [54–56]. It is composed primarily of chondrocytes that synthesize an abundant extracellular matrix under the direction of the chondrogenic transcription factors SOX9, L-SOX5, and SOX6 [57–60]. In addition, the extracellular matrix is specific for hyaline cartilage, half of which is made up of type II collagen, and different proteoglycans [60]. This composition confers on cartilage its viscoelastic property.

After cartilage injury (Figure 47.8), whether focal or widespread, the ability of native cartilage to heal itself is limited.

Historically, cartilage injuries have undergone a wide range of treatments, from a conservative approach to surgical



**Figure 47.8** A localized large posttraumatic chondral defect of the lateral femoral condyle of the right knee of a 32-year-old male patient as viewed by arthroscopy. Current surgical treatment includes microfracturing to induce bone marrow stem cell migration, leading to the formation of a fibrous cartilage substitute, or autologous chondrocyte transplantation. Stem/progenitor cell administration may be a future option for the treatment of localized cartilage defects. *Source:* courtesy of Dr J. Lützner.

interventions. Surgical modalities have included abrasion, drilling, microfracture, mosaicplasty, and autologous chondrocyte implantation, each of them having potential benefits and risks. (Mosaicplasty is a technique in which small circular (4–8 mm) autologous tissues are harvested from non-weight-bearing regions of the knee and then transplanted in a mosaic pattern until the osteochondral defect is filled.)

MSCs now are a promising cell source for cartilage regeneration that can be relatively easily obtained and then expanded in *ex vivo* culture to obtain high numbers of cells [61,62]. MSCs have been shown to have a chondrogenic differentiation potential when placed into chondrogenic media [11]. Johnstone *et al.* used the micro-mass culture system and centrifuged this into a condensed aggregate to which chondrogenic medium was added. Transforming growth factor (TGF)-β3 was the most important factor in the medium [63]. Other growth factors with the capacity to enhance the chondrogenic potential of MSCs have been identified, including BMPs [64], insulin-like growth factor-1 [65], and fibroblast growth factor-2 [66]. MSCs also have immune modulatory potential, including the ability to suppress the proliferation and activity of $CD4^+$ T cells, $CD8^+$ T cells, and memory T cells [67,68]. In spite of the potential advantages of MSCs in cartilage regeneration, there are some potential drawbacks to their use after chondrogenic differentiation, including expression of cartilage hypertrophy markers such as collagen type X, matrix metalloproteinase-13, parathyroid-hormone-related protein receptor, vascular endothelial growth factor (VEGF), and alkaline phosphatase, which could lead to ossification of the neocartilage tissue [69–72]. Additionally, regenerated cartilage has reduced thickness compared with mature native cartilage [73]. However, promising attempts at overcoming these hurdles have been made; for example, co-culture of MSCs with mature chondrocytes has been shown to reduce the expression of the hypertrophic markers [74]. Furthermore, addition of fibroblast-derived growth factor-2 and TGF-β1 to the MSC-induced cartilage tissue has also been shown to promote increased compressive stiffness, decreased surface tension, decreased water content, and increased collagen content – all characteristics of mature cartilage [75].

In an attempt to repair articular cartilage defects in goats, Guo *et al.* seeded culture-expanded MSCs derived from autologous bone marrow on bioceramic TCP scaffolds [76]. The study showed increased proteoglycan content of the neocartilage at 12–24 months after implantation. At 24 weeks the defects were resurfaced with a hyaline-like tissue with good incorporation into the normal cartilage and bone. Wakitani *et al.* treated full thickness articular cartilage defects on the weight-bearing surface of the medial femoral condyle of rabbits using bone marrow-derived MSCs [77]. The cells were seeded into a type I collagen gel before transplantation into the defect. Cell differentiation into chondrocytes was noted at 2 weeks, while complete regeneration of the defect without loss of overlying cartilage was documented at 24 weeks after transplantation. Yan and Yu transplanted MSC-seeded polylactic acid matrices in a full cartilage defect in rabbits and showed hyaline cartilage regeneration [78].

### 47.4.1 Osteoarthritis

MSCs have been the focus of attention in regenerating articular cartilage defects in OA in humans [79,80]. The first use of transplanted MSCs seeded on type I collagen gel to regenerate isolated, full-thickness articular cartilage defects in humans was reported by Wakitani *et al.* [79]. This study involved 24 patients with OA of the knees who had undergone tibial osteotomy. (Knee osteotomy is commonly used to realign arthritic damage on one side of the knee. The goal is to shift the patient's body weight off the damaged area to the other side of the knee where the cartilage is still healthy. Surgeons remove a wedge of the tibia from underneath the healthy side of the knee, which allows the tibia and femur to bend away from the damaged cartilage.) In the Wakitani *et al.* study 12 patients received bone-marrow-derived MSCs that were embedded in a collagen gel and transplanted on to the articular cartilage defect at the femoral condyle and covered with a periosteal flap, while the other 12 patients served as controls. At 42 weeks after transplantation the defects were covered with hyaline cartilage-like tissue in the MSC-transplanted group. Two years later, the treated group had a significantly greater amount of hyaline cartilage formation compared with the control group. In a later study the authors also demonstrated clinical and histological improvement in patients treated with autologous bone-marrow-derived MSCs for repair of articular cartilage defects in comparison with the control group [80]. Two patients with patellar cartilage defects were treated with cultured MSCs embedded in collagen gel and covered with autologous periosteum [80]. Even though histological analysis showed that the regenerated defect consisted of fibrocartilaginous new tissue, pain and walking ability improved 2 years postoperatively in the treatment group. Kuroda *et al.* reported on a 31-year-old male with a 20 mm × 30 mm full-thickness cartilage defect who was implanted with autologous bone-marrow-derived MSCs [81]. One year postoperatively there was significant improvement in clinical symptoms. Currently, there are 20 ongoing clinical trials registered at www.clinicaltrials.gov, recruiting patients to assess the efficacy of MSC-injection in OA, including a phase II multicenter study for OA of the knee [82]. However, clinical evidence of benefit in humans is still limited [82].

On the premise that articular cartilage defects are best repaired with autologous engineered cartilage tissue, there has been substantial research on ways to improve the outcome of cartilage regeneration [83]. These preclinical studies have focused on the efficacy of cartilage regeneration. However, the translation of these treatments into clinical practice has been slow [83].

## 47.5 Stem cells for tendon regeneration

Tendons are viscoelastic dense fibrous connective tissues that attach muscles to bone [84]. They are composed of fibroblasts, fibrocartilage cells, and occasionally fat cells. Collagen – especially type I – and cells constitute 85% of their dry weight,

water 55% of their wet weight, and proteoglycans <1% [84]. Tendon injuries can be divided into tendon rupture and tendinopathy [85]. Tendon injuries are common in athletes and people with jobs involving highly repetitive movements [86–89]. Irrespective of the type of injury, damaged tendons usually do not recover to the pre-injury status [85,90,91]. Tendinopathy, or overuse of tendons [88], has been described as the most frequent type of tendon injury, commonly found in the Achilles, patellar, shoulder rotator cuff, and forearm extensor tendons [92]. Various therapeutic modalities for treating tendon disorders include the use of nonsteroidal anti-inflammatory drugs [93–95], eccentric exercises [96–100], sclerosing injection treatment [101–104], low-intensity pulsed ultrasound [105,106], topical glyceryl trinitrate [18,107], shock-wave therapy [109–111], autografts, allografts, and prosthetic devices [112–114]. However, these approaches have had limited long-term benefits, and thus the development of tissue- or cell-engineered substitutes is being explored [84,115], including the use of MSCs [116]. MSCs have been shown in animal studies to significantly improve the structure and function of injured tendons [116,117]. Ohtera *et al.* conducted a study on the effect of fresh periosteum wrapped around tendons in a bone tunnel and found that the pullout strength of such tendons was significantly greater in the periosteal-tendon flap group than in a group that received frozen grafts 4 weeks postoperatively [118]. The authors concluded that the result was due to the presence of viable cells in the fresh periosteum. Goh *et al.* demonstrated the efficacy of bone-marrow-derived MSCs injected into the hallucis longus tendon using a calcaneal bone tunnel rabbit model [84]. Another study demonstrated structural and biomechanical improvement of Achilles tendons injected with allogeneic MSCs loaded on to a knitted poly-lactide-co-glycolide scaffold with fibrin glue [119]. These results were corroborated by two similar studies by Ouyang and co-workers [119,120]. A study by Awad *et al.* demonstrated a 15–40% increase in biomechanical properties of regenerated rabbit tendon defects implanted with bone-marrow-derived MSCs [116]. Young *et al.* suspended MSCs in a type I collagen gel on a biodegradable suture and then implanted the composite into rabbit Achilles tendon gap defects. Four weeks after implantation the MSC–collagen-grafted tendons were twice as stiff as the contralateral control tendons that received suture only [117]. Awad *et al.* implanted composites made from MSCs, collagen gels, and suture scaffolds into surgically created patellar tendon defects in rabbits [121]. The results at 12 and 26 weeks after surgery showed significant improvement in biomechanical properties compared with natural repair.

Animal and human studies have examined the use of MSCs in regeneration of rotator cuff tendon injury. The animal studies conducted by Chen *et al.* and Gullota *et al.* using MSC–collagen in a fibrin glue failed to show histological or biomechanical improvement [122,123]. However, in a clinical study by Ellera Gomes *et al.*, which included 14 patients with complete rotator cuff tears, the injured tendons were repaired and supplemented with autologous bone-marrow-derived MSCs [124]. The patients were monitored for 1 year. The University of California, Los Angeles scores were significantly improved in the treated patients, and healing was shown by MRI [125]. Although there was no control group in their study, the results demonstrated the potential benefit of MSCs in tendon repair [126]. In a small Phase I study, six patients with chronic Achilles tendinopathy refractory to conventionial treatment received an ultrasound-guided intratendinous injection of unrelated MHC-unmatched MSCs derived from term placenta. Symptomatic and functional improvement was rapid, and at 18 months after injection Doppler ultrasound imaging showed a marked decrease in the abnormal vasculature in the tendon compared with baseline, as well as slow regrowth of tendon tissue (Young *et al.*, unpublished data).

In a study by Smith and Webbon, in conjunction with VetCell Bioscience Ltd, more than 100 horses with acute tendon injuries had their tendons injected with bone-marrow-derived MSCs under tenoscopic and ultrasonographic control [91]. The results showed that the horses were back in full training after 48 weeks. However, the authors concluded that, despite the encouraging outcome, the efficacy for treating acute tendon lesions with MSCs has yet to be proven.

Although there may be potential benefits using MSC-based techniques in tendon regeneration, there are still fundamental issues that need to be resolved [116]. First, it has been observed that the majority of tissue-engineered tendons failed to reach the expected *in vivo* strength. Second, there are constraints in the clinical application of this technology by the interface between the newly developed tendon tissue and host bone. Third, the rate of tendon regeneration is often slow [84]. Future studies should explore (1) the use of mechanical stimulation, hormones, and growth factors to enhance the cellular differentiation process; (2) the cell concentration that prevents ectopic bone formation, and (3) the exploration of new matrices that can withstand the initial biomechanical loads after implantation [121].

## 47.6 Stem cells for skeletal muscle regeneration

After muscle injury, healing occurs in three different phases [127]. First, degeneration and inflammation continues for up to 2 weeks after the injury. Second, muscle regeneration begins 1 week after injury and peaks at about 14 days after the injury. Third, fibrosis occurs 2 weeks after the injury and increases over time until the fourth week after injury. The processes are interrelated and time dependent. There can be no full recovery if scar tissue occurs [127]. Thus, the goal of research in the regeneration of muscle is to optimize the process of regeneration and to minimize fibrosis. Satellite cells are essential for skeletal muscle regeneration. They are located in a niche on the surface of the muscle fiber beneath the ensheathing basal lamina [128]. During skeletal muscle healing satellite cells are activated under the influence of growth factors. They proliferate and differentiate into multinucleated myotubes and

eventually into regenerated myofibers [127]. Satellite cells have myogenic, adipogenic, and osteogenic differentiation potential. They are considered to be muscle-resident myogenic stem/progenitor cells [128].

Both acute and chronic muscle injuries lead to a systemic mobilization of MSCs increasing their number in the bloodstream [129]. Using a rat soleus muscle open crush model, Von Roth *et al.* demonstrated that the administration of $2.5 \times 10^6$ MSCs into the femoral artery significantly improved muscle forces (fast twitch and tetanus strength) after 3 weeks compared with the control group [130]. Nakabayashi *et al.* used a model of acute trauma to the tibialis anterior muscle in rats. They injected MSCs at the site of injury [131]. After 1 and 4 weeks muscle forces (fast twitch and tetanus strength) were significantly higher compared with the control group not given cells, and there was less than 50% fibrosis in the treated group. Using an external magnetic targeting system, the number of MSCs at the site of injury was increased, muscle force was enhanced, and fibrosis was reduced.

Muscle injury also occurs in systemic neurovascular diseases, and cell administration in these settings also showed improved outcomes after local [132,133] and systemic [134,135] administration of cells. A combination of insulin-like growth factor-1 and intravenous injection of MSCs in dystrophic mice enhanced muscle strength [134]. Jeong *et al.* differentiated induced pluripotent stem cells into MSCs and transplanted these cells locally into the tibialis anterior muscle of mice with a muscular dystrophy phenotype [133]. After 6 weeks, reduced levels of oxidative stress and increased dystrophin expression were detected in the MSC-treated group. Furthermore, the nuclei of transplanted MSCs were detected in the mouse muscle tissue.

## 47.7 Stem cells for wound repair

Injury and trauma initiate the mobilization of MSCs into the peripheral blood [136,137]. MSCs play a key role in wound healing, coordinating the repair response by recruiting cells and secreting growth factors as well as matrix proteins [138]. Wound healing is a complex process involving a series of coordinated events, including bleeding and coagulation, acute inflammation, cell migration, cell proliferation, cell differentiation, angiogenesis, re-epithelialization, and synthesis and remodeling of the extracellular matrix [138]. The process occurs in three overlapping phases in which MSCs play different roles: first, the inflammatory phase; second, the proliferative phase; and third, the remodeling phase [138].

The inflammatory phase begins after injury and lasts for 1–3 days. MSCs decrease secretion of the proinflammatory cytokines tumor necrosis factor-α and interferon-γ while increasing the production of the anti-inflammatory cytokines interleukin-10 and interleukin-4 [139]. The anti-inflammatory effect is particularly beneficial for chronic wound healing since MSCs can help shift the chronic inflammatory state to the next stage of healing [138,140,141]. Paracrine mechanisms reduce inflammation, promote angiogenesis, and induce cell migration and proliferation [142].

The proliferative phase starts on the third day after the trauma and lasts for approximately 2 weeks. MSCs enhance tissue repair by the production of growth factors (including VEGF, hepatocyte growth factor, and platelet-derived growth factor) and recruit keratinocytes, dermal fibroblasts, and host progenitor cells [138]. To restore the structure of the tissue, fibroblasts attach to the fibrin matrix and begin to produce collagen [143].

Remodeling is the final phase of wound healing and lasts 1–2 years or longer [144]. During this phase the degradation and remodeling processes lead to organized collagen bundles, resulting in better adaptation of the tissue to its function. MSCs produce soluble factors such as TGF-β3 and others that stimulate proliferation and migration of cells into the wound [138]. Paracrine signaling of MSCs provides antiscarring properties through secretion of VEGF and hepatocyte growth factor, as well as maintaining the balance between TGF-β1 and TGF-β3 [145–147].

Early clinical trials have shown enhanced healing of acute and chronic wounds treated with bone-marrow-derived MSCs injected or applied topically [148–152].

## 47.8 Conclusions

The potential of MSCs in the treatment of musculoskeletal and other disorders is promising. Preliminary data obtained from limited *in vitro* and *in vivo* studies are encouraging. Further preclinical and clinical studies demonstrating safety, efficacy, and cost effectiveness are necessary in order to substantiate a definitive role for MSCs in clinical practice.

## References

1 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.
2 Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**:393–403.
3 Wickham MQ, Erickson GR, Gimble JM, *et al.* Multipotent stromal cells derived from the infrapatellar fat pad of the knee. *Clin Orthop Relat Res* 2003;**412**:196–212.
4 Tuli R, Tuli S, Nandi S, *et al.* Characterization of multipotential mesenchymal progenitor cells derived from human trabecular bone. *Stem Cells* 2003;**21**:681–693.
5 Fukumoto T, Sperling JW, Sanyal A, *et al.* Combined effects of insulin-like growth factor-1 and transforming growth factor-β1 on periosteal mesenchymal cells during chondrogenesis in vitro. *Osteoarthritis Cartilage* 2003;**11**:55–64.

6 Okuno M, Muneta T, Koga H, *et al.* Meniscus regeneration by syngeneic, minor mismatched, and major mismatched transplantation of synovial mesenchymal stem cells in a rat model. *J Orthop Res* 2014;**32**:928–936.

7 Jankowski RJ, Deasy BM, Huard J. Muscle-derived stem cells. *Gene Ther* 2002;**9**:642–647.

8 Bakopoulou A, Leyhausen G, Volk J, *et al.* Comparative analysis of *in vitro* osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP). *Arch Oral Biol* 2011;**56**:709–721.

9 Liu Z-J, Zhuge Y, Velazquez OC. Trafficking and differentiation of mesenchymal stem cells. *J Cell Biochem* 2009;**106**:984–991.

10 Nixon AJ, Watts AE, Schnabel LV. Cell- and gene-based approaches to tendon regeneration. *J Shoulder Elb Surg* 2012;**21**: 278–294.

11 Baghaban Eslaminejad M, Malakooty Poor E. Mesenchymal stem cells as a potent cell source for articular cartilage regeneration. *World J Stem Cells* 2014;**6**:344–354.

12 Prockop DJ, Gregory CA, Spees JL. One strategy for cell and gene therapy: harnessing the power of adult stem cells to repair tissues. *Proc Natl Acad Sci U S A* 2003;**100**(Suppl 1):11917–11923.

13 Kasten P, Beyen I, Egermann M, Suda AJ, Moghaddam AA, Zimmermann G, *et al.* Instant stem cell therapy: characterization and concentration of human mesenchymal stem cells in vitro. *Eur. Cell. Mater.* 2008;**16**:47–55.

14 Mareschi K, Rustichelli D, Calabrese R, *et al.* Multipotent mesenchymal stromal stem cell expansion by plating whole bone marrow at a low cellular density: a more advantageous method for clinical use. *Stem Cells Int* 2012;**2012**:920581.

15 Fickert S, Schröter-Bobsin U, Gross A-F, *et al.* Human mesenchymal stem cell proliferation and osteogenic differentiation during long-term *ex vivo* cultivation is not age dependent. *J Bone Miner Metab* 2011;**29**:224–235.

16 Hu J, Zhou Y, Jiang D, Tan W. Proliferation and differentiation characteristics of human bone marrow mesenchymal stem cells during *ex-vivo* expansion. *J Cell Mol Immunol* 2006;**22**:7–10.

17 Robey PG, Kuznetsov SA, Ren J, *et al.* Generation of clinical grade human bone marrow stromal cells for use in bone regeneration. *Bone* 2015;**70**:87–92.

18 Jäger M, Jelinek EM, Wess KM, *et al.* Bone marrow concentrate: a novel strategy for bone defect treatment. *Curr Stem Cell Res Ther* 2009;**4**:34–43.

19 Gan Y, Dai K, Zhang P, *et al.* The clinical use of enriched bone marrow stem cells combined with porous β-tricalcium phosphate in posterior spinal fusion. *Biomaterials* 2008;**29**:3973–3982.

20 Hernigou P, Beaujean F. Treatment of osteonecrosis with autologous bone marrow grafting. *Clin Orthop* 2002;**405**:14–23.

21 Wakitani S, Nawata M, Tensho K, *et al.* Repair of articular cartilage defects in the patello-femoral joint with autologous bone marrow mesenchymal cell transplantation: three case reports involving nine defects in five knees. *J Tissue Eng Regen Med* 2007;**1**:74–79.

22 Horwitz EM, Prockop DJ, Fitzpatrick LA, *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999;**5**:309–313.

23 Horwitz EM, Gordon PL, Koo WKK, *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 2002;**99**:8932–8937.

24 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell* 2006;**126**:663–676.

25 Bruder SP, Kurth AA, Shea M, *et al.* Bone regeneration by implantation of purified, culture-expanded human mesenchymal stem cells. *J Orthop Res* 1998;**16**:155–162.

26 Arinzeh TL, Peter SJ, Archambault MP, *et al.* Allogeneic mesenchymal stem cells regenerate bone in a critical-sized canine segmental defect. *J Bone Joint Surg Am* 2003;**85-A**:1927–1935.

27 Nishikawa M, Myoui A, Ohgushi H, *et al.* Bone tissue engineering using novel interconnected porous hydroxyapatite ceramics combined with marrow mesenchymal cells: quantitative and three-dimensional image analysis. *Cell Transplant* 2004;**13**:367–376.

28 Kalia P, Blunn GW, Miller J, *et al.* Do autologous mesenchymal stem cells augment bone growth and contact to massive bone tumor implants? *Tissue Eng* 2006;**12**:1617–1626.

29 Korda M, Blunn G, Goodship A, Hua J. Use of mesenchymal stem cells to enhance bone formation around revision hip replacements. *J Orthop Res* 2008;**26**:880–885.

30 Bernstein P, Bornhauser M, Gunther KP, Stiehler M. Bone tissue engineering in clinical application: assessment of the current situation. *Orthopäde* 2009;**38**:1029–1037.

31 Giordano A, Galderisi U, Marino IR. From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells. *J Cell Physiol* 2007;**211**:27–35.

32 Zwingenberger S, Nich C, Valladares RD, *et al.* Recommendations and considerations for the use of biologics in orthopedic surgery. *BioDrugs* 2012;**26**:245–256.

33 Qin Y, Guan J, Zhang C. Mesenchymal stem cells: mechanisms and role in bone regeneration. *Postgrad Med J* 2014;**90**:643–647.

34 Ochs BG, Schmid U, Rieth J, *et al.* Acetabular bone reconstruction in revision arthroplasty: a comparison of freeze-dried, irradiated and chemically-treated allograft vitalised with autologous marrow versus frozen non-irradiated allograft. *J Bone Joint Surg Br* 2008;**90**:1164–1171.

35 Zwingenberger S, Yao Z, Jacobi A, *et al.* Stem cell attraction via SDF-1α expressing fat tissue grafts. *J Biomed Mater Res A* 2013;**101**:2067–2074.

36 Zwingenberger S, Yao Z, Jacobi A, *et al.* Enhancement of BMP-2 induced bone regeneration by SDF-1α mediated stem cell recruitment. *Tissue Eng Part A* 2014;**20**:810–818.

37 Pang X, Yang H, Peng B. Human umbilical cord mesenchymal stem cell transplantation for the treatment of chronic discogenic low back pain. *Pain Physician* 2014;**17**:E525–E530.

38 Yang W, Dong Y, Hong Y, *et al.* Evaluation of anterior vertebral interbody fusion using osteogenic mesenchymal stem cells transplanted in collagen sponge. *J Spinal Disord Tech* 2012; Epub ahead of print. doi: 10.1097/BSD.0b013e31825ca123.

39 Khashan M, Inoue S, Berven SH. Cell based therapies as compared to autologous bone grafts for spinal arthrodesis. *Spine* 2013;**38**: 1885–1891.

40 Houdek MT, Wyles CC, Martin JR, Sierra RJ. Stem cell treatment for avascular necrosis of the femoral head: current perspectives. *Stem Cells Cloning Adv Appl* 2014;**7**:65–70.

41 Arlet J, Ficat P. *Ischaemia and Necroses of Bone*. Williams & Wilkins: Baltimore, MD, 1980; pp. 75–102.

42 Ma Y, Wang T, Liao J, *et al.* Efficacy of autologous bone marrow buffy coat grafting combined with core decompression in patients

with avascular necrosis of femoral head: a prospective, double-blinded, randomized, controlled study. *Stem Cell Res Ther* 2014;**5**:115.

43 Gangji V, De Maertelaer V, Hauzeur J-P. Autologous bone marrow cell implantation in the treatment of non-traumatic osteonecrosis of the femoral head: five year follow-up of a prospective controlled study. *Bone* 2011;**49**:1005–1009.

44 Gangji V, Hauzeur J-P. Treatment of osteonecrosis of the femoral head with implantation of autologous bone-marrow cells. Surgical technique. *J Bone Joint Surg Am* 2005;**87**(Suppl 1):106–112.

45 Hernigou P, Daltro G, Filippini P, *et al.* Percutaneous implantation of autologous bone marrow osteoprogenitor cells as treatment of bone avascular necrosis related to sickle cell disease. *Open Orthop J* 2008;**2**:62–65.

46 Learmonth ID, Young C, Rorabeck C. The operation of the century: total hip replacement. *Lancet* 2007;**370**:1508–1519.

47 Gibon E, Yao Z, Rao AJ, *et al.* Effect of a CCR1 receptor antagonist on systemic trafficking of MSCs and polyethylene particle-associated bone loss. *Biomaterials* 2012;**33**:3632–3638.

48 Yao W, Lane NE. Targeted delivery of mesenchymal stem cells to the bone. *Bone* 2015;**70**:62–65.

49 Zhang XS, Linkhart TA, Chen S-T, *et al.* Local *ex vivo* gene therapy with bone marrow stromal cells expressing human BMP4 promotes endosteal bone formation in mice. *J Gene Med* 2004;**6**:4–15.

50 Rauch F, Glorieux FH. Osteogenesis imperfecta. *Lancet* 2004;**363**: 1377–1385.

51 Otsuru S, Gordon PL, Shimono K, *et al.* Transplanted bone marrow mononuclear cells and MSCs impart clinical benefit to children with osteogenesis imperfecta through different mechanisms. *Blood* 2012;**120**:1933–1941.

52 Oldershaw RA. Cell sources for the regeneration of articular cartilage: the past, the horizon and the future. *Int J Exp Pathol* 2012;**93**:389–400.

53 Temenoff JS, Mikos AG. Review: tissue engineering for regeneration of articular cartilage. *Biomaterials* 2000;**21**:431–440.

54 Demoor M, Ollitrault D, Gomez-Leduc T, *et al.* Cartilage tissue engineering: molecular control of chondrocyte differentiation for proper cartilage matrix reconstruction. *Biochim Biophys Acta* 2014;**1840**:2414–2440.

55 Hardingham T. Articular cartilage. In: Isenberg D, Maddison P, Woo P *et al.* (eds), *Oxford Textbook of Rheumatology*. Oxford University Press: Oxford, 2004; pp. 325–334.

56 Hardingham TE. Fell–Muir lecture: cartilage 2010 – the known unknowns. *Int J Exp Pathol* 2010;**91**:203–209.

57 Bell DM, Leung KK, Wheatley SC, *et al.* SOX9 directly regulates the type-II collagen gene. *Nat Genet* 1997;**16**:174–178.

58 Lefebvre V, Behringer RR, de Crombrugghe B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. *Osteoarthritis Cartilage* 2001;**9**(Suppl A):S69–S75.

59 Akiyama H, Chaboissier M-C, Martin JF, *et al.* The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. *Genes Dev* 2002;**16**:2813–2828.

60 Kuettner KE. Biochemistry of articular cartilage in health and disease. *Clin Biochem* 1992;**25**:155–163.

61 Caplan AI, Goldberg VM. Principles of tissue engineered regeneration of skeletal tissues. *Clin Orthop* 1999;**367** (Suppl):S12–S16.

62 Pittenger MF. Mesenchymal stem cells from adult bone marrow. *Methods Mol Biol* 2008;**449**:27–44.

63 Johnstone B, Hering TM, Caplan AI, *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**:265–272.

64 Steinert AF, Palmer GD, Pilapil C, *et al.* Enhanced in vitro chondrogenesis of primary mesenchymal stem cells by combined gene transfer. *Tissue Eng Part A* 2009;**15**:1127–1139.

65 Frisch J, Venkatesan JK, Rey-Rico A, *et al.* Influence of insulin-like growth factor I overexpression via recombinant adeno-associated vector gene transfer upon the biological activities and differentiation potential of human bone marrow-derived mesenchymal stem cells. *Stem Cell Res Ther* 2014;**5**:103.

66 Solchaga LA, Penick K, Porter JD, *et al.* FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol* 2005;**203**:398–409.

67 Le Blanc K, Rasmusson I, Götherström C, *et al.* Mesenchymal stem cells inhibit the expression of CD25 (interleukin-2 receptor) and CD38 on phytohaemagglutinin-activated lymphocytes. *Scand J Immunol* 2004;**60**:307–315.

68 Glennie S, Soeiro I, Dyson PJ, *et al.* Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005;**105**:2821–2827.

69 Sekiya I, Vuoristo JT, Larson BL, Prockop DJ. In vitro cartilage formation by human adult stem cells from bone marrow stroma defines the sequence of cellular and molecular events during chondrogenesis. *Proc Natl Acad Sci U S A* 2002;**99**:4397–4402.

70 Mwale F, Girard-Lauriault P-L, Wang HT, *et al.* Suppression of genes related to hypertrophy and osteogenesis in committed human mesenchymal stem cells cultured on novel nitrogen-rich plasma polymer coatings. *Tissue Eng* 2006;**12**:2639–2647.

71 Mwale F, Stachura D, Roughley P, Antoniou J. Limitations of using aggrecan and type X collagen as markers of chondrogenesis in mesenchymal stem cell differentiation. *J Orthop Res* 2006;**24**: 1791–1798.

72 Pelttari K, Winter A, Steck E, *et al.* Premature induction of hypertrophy during in vitro chondrogenesis of human mesenchymal stem cells correlates with calcification and vascular invasion after ectopic transplantation in SCID mice. *Arthritis Rheum* 2006;**54**:3254–3266.

73 Koga H, Engebretsen L, Brinchmann JE, *et al.* Mesenchymal stem cell-based therapy for cartilage repair: a review. *Knee Surg Sports Traumatol* 2009;**17**:1289–1297.

74 Bian L, Zhai DY, Mauck RL, Burdick JA. Coculture of human mesenchymal stem cells and articular chondrocytes reduces hypertrophy and enhances functional properties of engineered cartilage. *Tissue Eng Part A* 2011;**17**:1137–1145.

75 Khan IM, Francis L, Theobald PS, *et al. In vitro* growth factor-induced bio engineering of mature articular cartilage. *Biomaterials* 2013;**34**:1478–1487.

76 Guo X, Wang C, Duan C, *et al.* Repair of osteochondral defects with autologous chondrocytes seeded onto bioceramic scaffold in sheep. *Tissue Eng* 2004;**10**:1830–1840.

77 Wakitani S, Goto T, Pineda SJ, *et al.* Mesenchymal cell-based repair of large, full-thickness defects of articular cartilage. *J Bone Joint Surg Am* 1994;**76**:579–592.

78 Yan H, Yu C. Repair of full-thickness cartilage defects with cells of different origin in a rabbit model. *Arthroscopy* 2007;**23**:178–187.

79 Wakitani S, Imoto K, Yamamoto T, *et al.* Human autologous culture expanded bone marrow mesenchymal cell transplantation for repair of cartilage defects in osteoarthritic knees. *Osteoarthritis Cartilage* 2002;**10**:199–206.

80 Wakitani S, Mitsuoka T, Nakamura N, *et al.* Autologous bone marrow stromal cell transplantation for repair of full-thickness articular cartilage defects in human patellae: two case reports. *Cell Transplant* 2004;**13**:595–600.

81 Kuroda R, Ishida K, Matsumoto T, *et al.* Treatment of a full-thickness articular cartilage defect in the femoral condyle of an athlete with autologous bone-marrow stromal cells. *Osteoarthritis Cartilage* 2007;**15**:226–231.

82 Wang Y, Yuan M, Guo QY, *et al.* Mesenchymal stem cells for treating articular cartilage defects and osteoarthritis. *Cell Transplant* 2015;**24**:1661–1678.

83 Chiang H, Jiang CC. Repair of articular cartilage defects: review and perspectives. *J Formos Med Assoc* 2009;**108**:87–101.

84 Goh JCH, Ouyang HW, Teoh SH, *et al.* Tissue-engineering approach to the repair and regeneration of tendons and ligaments. *Tissue Eng* 2003;**9**(Suppl 1):S31–S44.

85 Sharma P, Maffulli N. Tendon injury and tendinopathy: healing and repair. *J Bone Joint Surg Am* 2005;**87**:187–202.

86 Longo UG, Rittweger J, Garau G, *et al.* No influence of age, gender, weight, height, and impact profile in Achilles tendinopathy in Masters track and field athletes. *Am J Sports Med* 2009;**37**:1400–1405.

87 Hess GW. Achilles tendon rupture: a review of etiology, population, anatomy, risk factors, and injury prevention. *Foot Ankle Spec* 2010;**3**:29–32.

88 Maffulli N, Khan KM, Puddu G. Overuse tendon conditions: time to change a confusing terminology. *Arthroscopy* 1998;**14**:840–843.

89 Maffulli N, Wong J, Almekinders LC. Types and epidemiology of tendinopathy. *Clin Sports Med* 2003;**22**:675–692.

90 Dowling BA, Dart AJ, Hodgson DR, Smith RK. Superficial digital flexor tendonitis in the horse. *Equine Vet J* 2000;**32**:369–378.

91 Smith RKW, Webbon PM. Harnessing the stem cell for the treatment of tendon injuries: heralding a new dawn? *Br J Sports Med* 2005;**39**:582–584.

92 Rees JD, Wilson AM, Wolman RL. Current concepts in the management of tendon disorders. *Rheumatol Oxf Engl* 2006;**45**:508–521.

93 Aström M, Westlin N. No effect of piroxicam on achilles tendinopathy. A randomized study of 70 patients. *Acta Orthop Scand* 1992;**63**:631–634.

94 Auclair J, Georges J, Grapton X. A double-blind controlled multicenter study of percutaneous niflumic acid gel and placebo in the treatment of Achilles heel tendinitis. *Curr Ther Res* 1989;**46**:782–788.

95 Jakobsen T, Petersen L, Christiansen S, Haarbo J. Tenoxicam vs placebo in the treatment of tendinitis, periostitis, and sprains. *Curr Ther Res* 1989;**45**:213–220.

96 Roos EM, Engström M, Lagerquist A, Söderberg B. Clinical improvement after 6 weeks of eccentric exercise in patients with mid-portion Achilles tendinopathy – a randomized trial with 1-year follow-up. *Scand J Med Sci Sports* 2004;**14**:286–295.

97 Petersen W, Welp R, Rosenbaum D. Chronic Achilles tendinopathy: a prospective randomized study comparing the therapeutic effect of eccentric training, the AirHeel brace, and a combination of both. *Am J Sports Med* 2007;**35**:1659–1667.

98 Frohm A, Saartok T, Halvorsen K, Renström P. Eccentric treatment for patellar tendinopathy: a prospective randomised short-term pilot study of two rehabilitation protocols. *Br J Sports Med* 2007;**41**:e7.

99 Rompe JD, Furia J, Maffulli N. Eccentric loading versus eccentric loading plus shock-wave treatment for midportion Achilles tendinopathy: a randomized controlled trial. *Am J Sports Med* 2009;**37**:463–470.

100 De Jonge S, de Vos RJ, Van Schie HTM, *et al.* One-year follow-up of a randomised controlled trial on added splinting to eccentric exercises in chronic midportion Achilles tendinopathy. *Br J Sports Med* 2010;**44**:673–677.

101 Willberg L, Sunding K, Ohberg L, *et al.* Sclerosing injections to treat midportion Achilles tendinosis: a randomised controlled study evaluating two different concentrations of polidocanol. *Knee Surg Sports Traumatol Arthrosc* 2008;**16**:859–864.

102 Knobloch K. Sclerosing polidocanol injections in Achilles tendinopathy in high level athletes. *Knee Surg Sports Traumatol Arthrosc* 2008;**16**:1061–1063.

103 Willberg L, Sunding K, Forssblad M, *et al.* Sclerosing polidocanol injections or arthroscopic shaving to treat patellar tendinopathy/jumper's knee? A randomised controlled study. *Br J Sports Med* 2011;**45**:411–415.

104 Clementson M, Lorén I, Dahlberg L, Aström M. Sclerosing injections in midportion Achilles tendinopathy: a retrospective study of 25 patients. *Knee Surg Sports Traumatol Arthrosc* 2008;**16**:887–890.

105 Warden SJ, Avin KG, Beck EM, *et al.* Low-intensity pulsed ultrasound accelerates and a nonsteroidal anti-inflammatory drug delays knee ligament healing. *Am J Sports Med* 2006;**34**:1094–1102.

106 Warden SJ. A new direction for ultrasound therapy in sports medicine. *Sports Med* 2003;**33**:95–107.

107 Paoloni JA, Appleyard RC, Nelson J, Murrell GAC. Topical glyceryl trinitrate treatment of chronic noninsertional Achilles tendinopathy. A randomized, double-blind, placebo-controlled trial. *J Bone Joint Surg Am* 2004;**86-A**:916–922.

108 Hunte G, Lloyd-Smith R. Topical glyceryl trinitrate for chronic Achilles tendinopathy. *Clin J Sport Med* 2005;**15**:116–117.

109 Speed CA, Nichols D, Richards C, *et al.* Extracorporeal shock wave therapy for lateral epicondylitis – a double blind randomised controlled trial. *J Orthop Res* 2002;**20**:895–898.

110 Speed CA, Richards C, Nichols D, *et al.* Extracorporeal shock-wave therapy for tendonitis of the rotator cuff. A double-blind, randomised, controlled trial. *J Bone Joint Surg Br* 2002;**84**:509–512.

111 Seil R, Wilmes P, Nührenbörger C. Extracorporeal shock wave therapy for tendinopathies. *Expert Rev Med Devices* 2006;**3**:463–470.

112 Kato YP, Dunn MG, Zawadsky JP, *et al.* Regeneration of Achilles tendon with a collagen tendon prosthesis. Results of a one-year implantation study. *J Bone Joint Surg Am* 1991;**73**:561–574.

113 Steenbrugge F, Verdonk R, Vorlat P, *et al.* Repair of chronic ruptures of the anterior cruciate ligament using allograft reconstruction and a ligament augmentation device. *Acta Orthop Belg* 2001;**67**:252–258.

114 Jørgensen U, Bak K, Ekstrand J, Scavenius M. Reconstruction of the anterior cruciate ligament with the iliotibial band autograft in patients with chronic knee instability. *Knee Surg Sports Traumatol Arthrosc* 2001;**9**:137–145.

115 Langer R, Vacanti JP. Tissue engineering. *Science* 1993;**260**:920–926.

116 Awad HA, Butler DL, Boivin GP, *et al.* Autologous mesenchymal stem cell-mediated repair of tendon. *Tissue Eng* 1999;**5**:267–277.

117 Young RG, Butler DL, Weber W, *et al.* Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. *J Orthop Res* 1998;**16**:406–413.

118 Ohtera K, Yamada Y, Aoki M, *et al.* Effects of periosteum wrapped around tendon in a bone tunnel: a biomechanical and histological study in rabbits. *Crit Rev Biomed Eng* 2000;**28**:115–118.

119 Ouyang HW, Goh JCH, Thambyah A, *et al.* Knitted poly-lactide-co-glycolide scaffold loaded with bone marrow stromal cells in repair and regeneration of rabbit Achilles tendon. *Tissue Eng* 2003;**9**:431–439.

120 Ouyang HW, Goh JCH, Mo XM, *et al.* The efficacy of bone marrow stromal cell-seeded knitted PLGA fiber scaffold for Achilles tendon repair. *Ann N Y Acad Sci* 2002;**961**:126–129.

121 Awad HA, Boivin GP, Dressler MR, *et al.* Repair of patellar tendon injuries using a cell-collagen composite. *J Orthop Res* 2003;**21**: 420–431.

122 Chen JM, Willers C, Xu J, *et al.* Autologous tenocyte therapy using porcine-derived bioscaffolds for massive rotator cuff defect in rabbits. *Tissue Eng* 2007;**13**:1479–1491.

123 Gulotta LV, Kovacevic D, Ehteshami JR, *et al.* Application of bone marrow-derived mesenchymal stem cells in a rotator cuff repair model. *Am J Sports Med* 2009;**37**:2126–2133.

124 Ellera Gomes JL, da Silva RC, Silla LMR, *et al.* Conventional rotator cuff repair complemented by the aid of mononuclear autologous stem cells. *Knee Surg Sports Traumatol Arthrosc* 2012;**20**:373–377.

125 Longo UG, Berton A, Ahrens PM, *et al.* Clinical tests for the diagnosis of rotator cuff disease. *Sports Med Arthrosc Rev* 2011;**19**:266–278.

126 Longo UG, Rizzello G, Berton A, *et al.* Biological strategies to enhance rotator cuff healing. *Curr Stem Cell Res Ther* 2013;**8**: 464–470.

127 Huard J, Li Y, Fu FH. Muscle injuries and repair: current trends in research. *J Bone Joint Surg Am* 2002;**84-A**:822–832.

128 Relaix F, Zammit PS. Satellite cells are essential for skeletal muscle regeneration: the cell on the edge returns centre stage. *Dev Camb Engl* 2012;**139**:2845–2856.

129 Ramírez M, Lucia A, Gómez-Gallego F, *et al.* Mobilisation of mesenchymal cells into blood in response to skeletal muscle injury. *Br J Sports Med* 2006;**40**:719–722.

130 Von Roth P, Duda GN, Radojewski P, *et al.* Intra-arterial MSC transplantation restores functional capacity after skeletal muscle trauma. *Open Orthop J* 2012;**6**:352–356.

131 Nakabayashi A, Kamei N, Sunagawa T, *et al. In vivo* bioluminescence imaging of magnetically targeted bone marrow-derived mesenchymal stem cells in skeletal muscle injury model. *J Orthop Res* 2013;**31**:754–759.

132 Shabbir A, Zisa D, Leiker M, *et al.* Muscular dystrophy therapy by nonautologous mesenchymal stem cells: muscle regeneration without immunosuppression and inflammation. *Transplantation* 2009;**87**:1275–1282.

133 Jeong J, Shin K, Lee SB, *et al.* Patient-tailored application for Duchene muscular dystrophy on mdx mice based induced mesenchymal stem cells. *Exp Mol Pathol* 2014;**97**:253–258.

134 Secco M, Bueno C, Vieira NM, *et al.* Systemic delivery of human mesenchymal stromal cells combined with IGF-1 enhances muscle functional recovery in LAMA2 dy/2j dystrophic mice. *Stem Cell Rev* 2013;**9**:93–109.

135 De Bari C, Dell'Accio F, Vandenabeele F, *et al.* Skeletal muscle repair by adult human mesenchymal stem cells from synovial membrane. *J Cell Biol* 2003;**160**:909–918.

136 Mansilla E, Marín GH, Drago H, *et al.* Bloodstream cells phenotypically identical to human mesenchymal bone marrow stem cells circulate in large amounts under the influence of acute large skin damage: new evidence for their use in regenerative medicine. *Transplant Proc* 2006;**38**:967–969.

137 Wang Y, Johnsen HE, Mortensen S, *et al.* Changes in circulating mesenchymal stem cells, stem cell homing factor, and vascular growth factors in patients with acute ST elevation myocardial infarction treated with primary percutaneous coronary intervention. *Heart* 2006;**92**:768–774.

138 Maxson S, Lopez EA, Yoo D, *et al.* Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med* 2012;**1**:142–149.

139 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.

140 Newman RE, Yoo D, LeRoux MA, Danilkovitch-Miagkova A. Treatment of inflammatory diseases with mesenchymal stem cells. *Inflamm Allergy Drug Targets* 2009;**8**:110–123.

141 Singer NG, Caplan AI. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol* 2011;**6**:457–478.

142 Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ Res* 2008;**103**:1204–1219.

143 Clark RA. Fibrin and wound healing. *Ann N Y Acad Sci* 2001;**936**:355–367.

144 Ramasastry SS. Acute wounds. *Clin Plast Surg* 2005;**32**:195–208.

145 Ono I, Yamashita T, Hida T, *et al.* Combined administration of basic fibroblast growth factor protein and the hepatocyte growth factor gene enhances the regeneration of dermis in acute incisional wounds. *Wound Repair Regen* 2004;**12**:67–79.

146 Shah M, Foreman DM, Ferguson MW. Neutralisation of TGF-β1 and TGF-β2 or exogenous addition of TGF-β3 to cutaneous rat wounds reduces scarring. *J Cell Sci* 1995;**108**:985–1002.

147 Colwell AS, Beanes SR, Soo C, *et al.* Increased angiogenesis and expression of vascular endothelial growth factor during scarless repair. *Plast Reconstr Surg* 2005;**115**:204–212.

148 Badiavas EV, Falanga V. Treatment of chronic wounds with bone marrow-derived cells. *Arch Dermatol* 2003;**139**:510–516.

149 Falanga V, Iwamoto S, Chartier M, *et al.* Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. *Tissue Eng* 2007;**13**:1299–1312.

150 Yoshikawa T, Mitsuno H, Nonaka I, *et al.* Wound therapy by marrow mesenchymal cell transplantation. *Plast Reconstr Surg* 2008;**121**:860–877.

151 Dash NR, Dash SN, Routray P, *et al.* Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuvenation Res* 2009;**12**:359–366.

152 Lu D, Chen B, Liang Z, *et al.* Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res Clin Pract* 2011;**92**:26–36.

CHAPTER 48

# The potential role of bone marrow mesenchymal stromal cells in the treatment of ischemic stroke

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## 48.1 Introduction

### 48.1.1 Stroke

In the 1970s the World Health Organization defined stroke as a neurological deficit of cerebrovascular cause that persists beyond 24 h or is interrupted by death within 24 h. A stroke, also known as a cerebrovascular accident, occurs is when the brain is damaged either by poor blood flow to the brain or by hemorrhage into the brain, both resulting in death of brain cells. Strokes result in part or parts of the brain not performing normally and can lead to major functional neurological deficits such as hemiparesis – paralysis of one side of the body, a condition first noted by Hippocrates (Figure 48.1).

There are two main types of stroke: ischemic stroke due to lack of blood flow to the brain and hemorrhagic stroke due to bleeding into the brain or into the ventricular system within the brain, or both. Ischemic strokes are caused by interruption of the blood supply to the brain, while hemorrhagic strokes result from the rupture of a blood vessel or an abnormal vascular structure in the brain such as an arterial aneurysm. A schema depicting how a cerebral hemorrhage can occur from such a vascular abnormality is shown in Figure 48.2.

About 87% of strokes are ischemic, the rest being hemorrhagic. Hemorrhagic strokes can be associated with a severe headache as well as severe functional impairment [1]. A computed tomography (CT) scan showing brain hemorrhage is shown in Figure 48.3.

This chapter deals only with ischemic stroke. In an ischemic stroke, blood supply to part of the brain is decreased, leading to dysfunction of the brain tissue in that area. This causes cerebral infarction. There are three main ways by which this can happen:

1. Thrombosis – obstruction of a cerebral artery by a blood clot forming locally.
2. Embolism – obstruction of a cerebral artery due to an embolus from elsewhere in the body, such as the heart in atrial fibrillation.
3. Systemic hypoperfusion – underperfusion of the arterial blood supply to the brain (e.g., due to low blood pressure, as can occur in severe infection or severe hemorrhage).

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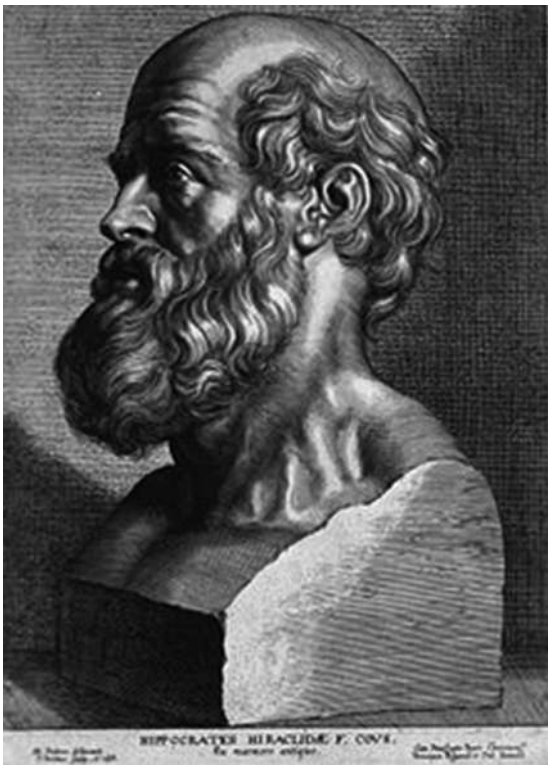


**Figure 48.1** An engraving of Hippocrates by Peter Paul Rubens made in 1638. Courtesy of the USA National Library of Medicine. Hippocrates (circa 460–circa 370 BC) was a Greek physician in the age of Pericles (Classical Greece) and is considered one of the most outstanding figures in the history of medicine. He first described the sudden paralysis that is often associated with stroke. He is referred to as the "Father of Western Medicine." *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain (PD 1923). Its attribution is as follows: "Hippocrates rubens." Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Hippocrates_rubens.jpg#/media/File:Hippocrates_rubens.jpg.

Ischemic stroke is a leading cause of death in the world [2] and also one of the primary causes for adult-acquired disability [1,3]. Signs and symptoms of a stroke may include an inability to move or problems in understanding or speaking, feeling like the world is spinning, or loss of vision on one side, among others. Signs and symptoms often appear soon after the stroke has occurred. The symptoms of a stroke can be permanent. If symptoms last less than 1 or 2 h it is known as a transient ischemic attack.

The diagnosis of stroke is typically made clinically and confirmed with medical imaging such as CT scanning or magnetic resonance imaging (MRI). Optimal treatment utilizes recombinant tissue plasminogen activator (tPA) to lyse the thrombus or embolism causing an ischemic stroke [4–7].

In 2010, approximately 17 million people had a stroke and 33 million people had previously had a stroke and were still alive. Between 1990 and 2010 the number of strokes that occurred each year decreased by approximately 10% in the developed world and increased by 10% in the developing world [8]. In 2013, stroke was the second most frequent cause of death after coronary artery disease, accounting for 6.4 million deaths (12% of the total) [9]. About 3.3 million deaths resulted from ischemic stroke, while 3.2 million deaths resulted from hemorrhagic stroke [9]. About half of people who have had a stroke live less than 1 year [3]. Overall, two-thirds of strokes occurred in those over 65 years of age.

Currently, recombinant tPA is the major pharmacologic agent used to treat acute ischemic stroke [4], but its efficacy is limited by a short therapeutic time window for administration of only 4.5 h from the time of the stroke event [5]. In the USA, only 5% of patients with acute ischemic stroke receive therapy with tPA [6].

Even when thrombolytic therapy or percutaneous vascular intervention is administered in time, many patients still experience deficits in neurological function. Currently, the primary treatment goals for ischemic stroke are recovery of cerebral perfusion in the acute stage and promotion of reestablishment of the cerebral circulation to avoid death of brain cells [7]. Comparatively little attention has been paid to enhancing long-term repair and recovery of brain function after ischemic stroke. Current thinking in treatment is guided by the principle of prophylaxis, with a focus on avoiding risk factors for cerebrovascular disease by developing healthy living habits and taking drugs such as statins prophylactically to prevent the occurrence of ischemic stroke. More recently, cell therapy has been studied in the treatment of ischemic stroke.

### 48.1.2 Stem/stromal cells

In the treatment of cerebral ischemic disease stem/stromal cells provide trophic support, enhance angiogenesis, replace dead neurons or stimulate the regeneration of endogenous cells in the ischemic area [8]. A number of different types of cell have been listed as candidates for cell therapy of cerebral ischemia, including embryonic stem cells, neural stem cells, induced pluripotent stem cells, umbilical cord blood cells, and bone-marrow-derived mesenchymal stromal cells (BM-MSCs) [9]. BM-MSCs appear especially promising as a potential avenue for treatment for several reasons: they are relatively easy to obtain from the patient's own body and they can be expanded in numbers by *in vitro* culture. They do not pose ethical issues or cause immunological side effects [10].

Bone-marrow-derived stem/progenitor cells include BM-MSCs [11], hematopoietic stem cells [12], and endothelial progenitor cells [13]. Hematopoietic stem cells are the stem cells for the hematopoietic and immune systems [13]. Endothelial progenitor cells play an important role in angiogenesis, and may differentiate into endothelial cells [14].

### 48.1.3 Mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) can be derived from a wide variety of tissues, including bone marrow, adipose tissue, umbilical cord blood, Wharton's jelly, placental tissue, tooth buds, and hepatic tissue [15,16]. MSCs can differentiate into cells of mesodermal lineage, and under certain conditions they are capable of differentiating into cells that have the phenotypic

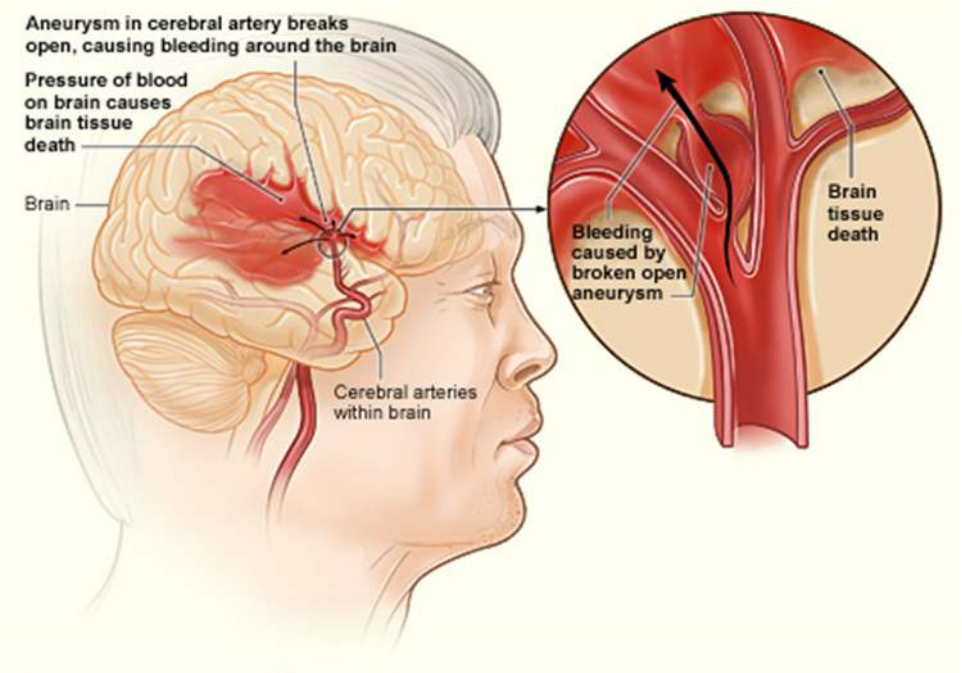



**Figure 48.2** The illustration shows how a hemorrhagic stroke can occur in the brain. An aneurysm in a cerebral artery breaks open, which causes bleeding in the brain. The pressure of the blood causes brain tissue death. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain because it has been placed there by its creator. The attribution is as follows: "Stroke hemorrhagic" by National Heart Lung and Blood Institute (NIH) – National Heart Lung and Blood Institute (NIH). Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Stroke_hemorrhagic.jpg#/media/File:Stroke_hemorrhagic.jpg.

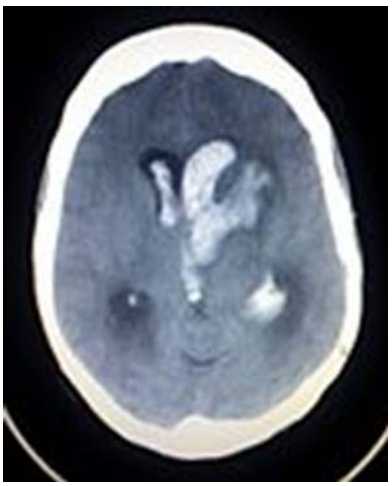


**Figure 48.3** A CT image of the brain showing intracerebral and intraventricular hemorrhages in a young woman. The white areas in the image represent blood in the ventricular system of the brain and on both sides of the brain. *Source:* https://en.wikipedia.org/wiki/File:Intracerebral_heamorrage_2.jpg.

characteristics of neurons, glial cells, and endothelial cells [11,17,18], although there are few reports that after phenotypic differentiation to neural cells they can *function* as neural cells. Human BM-MSCs do not normally express markers of hematopoietic cells, such as CD11, CD14, CD34, or CD45, or costimulatory molecules such as CD40 ligand (CD154), CD80, or CD86. BM-MSCs can express CD105, CD73, CD44, CD90 (Thy-1), CD71, and stromal precursor antigen-1, as well as the adhesion molecules CD106, CD166, intercellular adhesion molecular-1, and CD29 [19]. However, no specific markers for BM-MSCs have yet been identified. Differences in the methods used for isolation and expansion can affect the expression of the surface markers of BM-MSCs [11,20–24] (and see Chapter 7).

As indicated previously, cerebrovascular disease is one of the major causes of death worldwide [25], and it is possible that BM-MSCs can provide therapeutic benefit after an ischemic stroke. Regenerative processes are usually initiated in the brain within a few days to a few weeks after the occurrence of cerebral ischemia, providing a window of opportunity for the use BM-MSCs [26]. We suggest that these neurorestorative processes may include trophic support, angiogenesis, synaptic plasticity, and neurogenesis and contribute to functional recovery [27]. In experiments using animal models of cerebral ischemia it has been found that the ischemic cerebral tissue induces BM-MSCs to migrate into the ischemic area [28]. BM-MSCs secrete cytokines and trophic factors [29], promote angiogenesis and neurogenesis [30,31], and regulate the acute and chronic pathological process of ischemic stroke by means of anti-inflammatory, antiapoptotic, and immunoregulatory mechanisms [11].

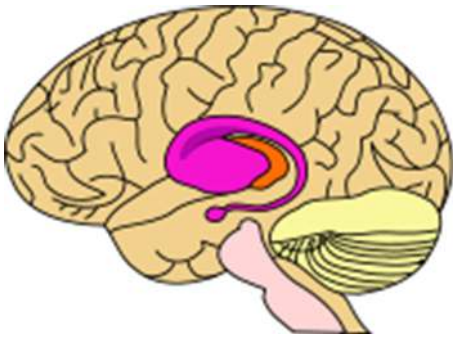


**Figure 48.4** Structures of brain: the caudate nucleus and putamen, and the thalamus. *Source:* https://commons.wikimedia.org/wiki/File:BrainCaudatePutamen.svg#/media/File:BrainCaudatePutamen.svg.

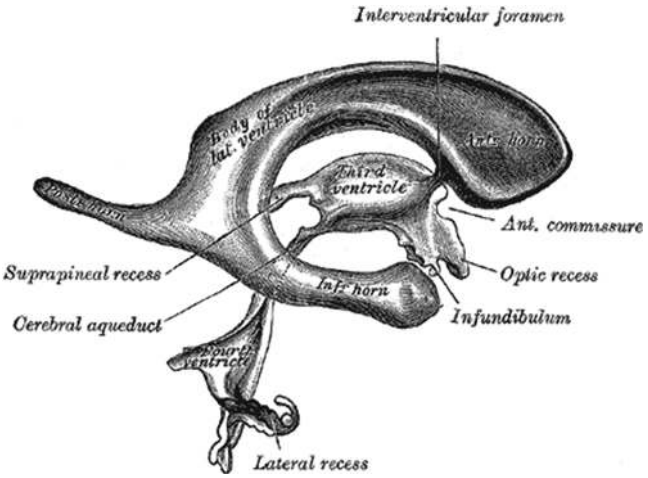



**Figure 48.5** Drawing of a cast of the ventricular cavities, viewed from the side. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain (PD 1923). The author was Henry Vandyke Carter (1831–1897) and the source was Henry Gray (1918) *Anatomy of the Human Body*. The attribution is as follows: "Gray736" by Henry Vandyke Carter – Henry Gray (1918) *Anatomy of the Human Body* (See "Book" section below) Bartleby.com: Gray's Anatomy, Plate 736. Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Gray736.png#/media/File:Gray736.png.

BM-MSCs can be transplanted in animal experiments by either stereotactically guided intracranial (IC) injection or by intravascular injection [32]. Several clinical trials have administered BM-MSCs to ischemic stroke patients and the preliminarily findings have suggested that the transplantation of BM-MSCs in a clinical setting is safe and feasible [33–36].

## 48.2 Transplantation route and mechanisms of migration

BM-MSC administration by the IC route can be into the striatum or into the lateral ventricle [37]. The striatum is divided by a white matter tract called the internal capsule into two sectors called the caudate nucleus and the putamen (Figure 48.4). The ventricular system is a set of four interconnected cavities (ventricles) in the brain, where the cerebrospinal fluid (CSF) is produced (Figures 48.5 and 48.6). The intravascular route can be by either intra-arterial (IA) or intravenous (IV) injection [38].

In early studies MSCs were transplanted intracranially by directly injecting them into the injured brain or into the nearby ventricular system. Several studies have shown that the same therapeutic effects can be achieved by administering BM-MSCs outside the central nervous system (CNS) by IA or IV injection [38]. Neurological function improved after transplantation

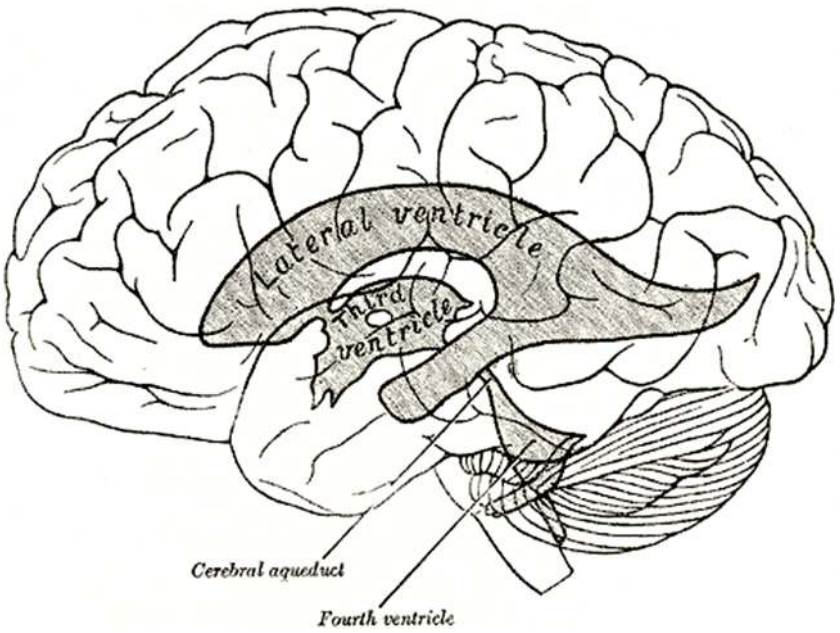



**Figure 48.6** Scheme showing relations of the ventricles to the surface of the brain. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain (PD 1923). The author was Henry Vandyke Carter (1831–1897) and the source was Henry Gray (1918) *Anatomy of the Human Body*. The attribution is as follows: "Gray734" by Henry Vandyke Carter – Henry Gray (1918) *Anatomy of the Human Body* (See "Book" section below) Bartleby.com: Gray's Anatomy, Plate 734. Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Gray734.png#/media/File:Gray734.png.

of BM-MSCs by different routes and at different times in experiments using animal models of cerebral ischemia. However, there is still a lack of a comprehensive and thorough comparison between the various routes of BM-MSC transplantation (Table 48.1).

Transplantation directly into the striatum may be the best way of targeting of BM-MSCs to the ischemic area of the brain [80]. BM-MSCs transplanted into the striatum reduce NVU disruption caused by tPA after MCAO [62] (see legend of Table 48.1 for abbreviations). The MCA is the artery most often occluded in

**Table 48.1** Optimum delivery routes and timing of MSC transplantation in animal models of ischemic stroke.

| Stroke model | Delivery route | Timing | Results | Reference |
|---|---|---|---|---|
| Permanent MCAO | IC (striatum) | 3/4/7 days | MSCs survived, migrated toward IBZ, expressed neuronal or astrocytic markers, increased axonal sprouting, produced many trophic factors, and remyelination occurred in the cortical penumbra | [39–42] |
| Transient MCAO (2 h) | IC (IBZ) | 24 h/3 days | Enhanced endogenous neurogenesis; increased MMP-9 activation, stimulation of neurons to modulate CBF | [30,43] |
| Transient MCAO (2 h)/ permanent MCAO | IC (striatum) | 24 h/7 days | Inhibited pathologic upregulation of brain-isoform glucose transporters types 1 and 3, and promoted recovery of local glucose metabolism in peri-infarct area | [44,45] |
| Permanent MCAO | IC (lateral ventricle) | 24 h | MSCs survived, adhered to ventricular wall, and reduced behavioral functional deficits | [46] |
| Cortical ischemia | IC | 7 days | Human MSCs survived, expressed markers of neuroectodermal cells and ameliorated neurological deficits | [47] |
| Transient MCAO (2 h) | IA | 24 h | MSCs distributed throughout the area of MCA, upregulated the expression of BMP2 and BMP4, gap junction protein CX-43, and synaptophysin | [48,49] |
| Transient MCAO (2 h) | IA | 24 h/2 h | Reduced lesion size, increased axon–myelin remodeling | [50,51] |
| Transient MCAO (2 h) | IV | 24 h | Produced many trophic factors; MCP-1 levels significantly increased from 6 h, peaked at 48 h after MCAO, and enhanced MSC migration and tissue repair | [43,52] |
| Transient MCAO (2 h)/ permanent MCAO | IV | 24 h | Human MSCs expressed bFGF and VEGF, promoted expression of IGF-1 and receptor, which contributed to improved recovery and increased neurogenesis | [29,53,54] |
| Transient MCAO (2 h) | IV | 24 h | Cell-free MSC-derived exosomes survived, enhanced neurite remodeling and angiogenesis, and improved functional recovery; male MSCs increased bFGF expression, reduced apoptosis, and improved functional recovery in female rats | [55,56] |
| Transient MCAO (90 min) | IV | 3 h | Downregulated expression of survivin and Bcl-2, prevented apoptosis and cell death | [57] |
| Global cerebral ischemia | IV | 3 h | Reduced infarct volume and neuronal loss, increased BDNF expression, ameliorated functional deficits | [58] |
| Lacunar stroke | IC (striatum) | 7 days | Enhanced the recovery of motor function, migrated towards the lesion, enhanced neurogenesis, expressed neuronal or astrocytic phenotypes | [59] |
| Permanent MCAO | IV (tail vein) | 3 h | MSCs reduced the infarct size, improved functional deficits, attenuated IL-23/IL-17 expression | [60] |
| Barrel cortex stroke | IC | 1/7 days | Restored the thalamocortical circuit connections; increased the density of neurons, axons, and blood vessels; enhanced local blood flow; produced cytokines/trophic factors | [61] |
| Transient MCAO (90 min) | IC | 24 h | MSCs ameliorated MMP-9 activation, improved NVU disruption | [62] |
| SHR-SP | IC (striatum) | 8 weeks | MSCs ameliorated ventricular dilatation, reduced the breakdown of neurovascular integrity | [63] |
| Permanent MCAO | IC/IV | 7 days | MSCs enhanced functional recovery (IC), expressed neural cell makers (IV) | [64,65] |
| Transient MCAO (2 h) | IV (tail vein) | 24 h | MSCs produced cytokines (GDNF) | [66] |

**Table 48.1** (*Continued*)

| Stroke model | Delivery route | Timing | Results | Reference |
|---|---|---|---|---|
| Permanent MCAO | IV | 1 day | Enhanced functional recovery, increased axonal density in both contralateral motor cortex and ipsilateral rostral forelimb area, enhanced interhemispheric and intracortical connections, regulated cytokines (TGF-β) | [67,68] |
| Transient MCAO (60 min) | IA | 2/7 days | MSCs did not improve functional recovery, but enhanced angiogenesis | [69] |
| Transient MCAO (2 h) | IC (striatum) | 3 days | MSCs secreted TGF-β to suppress immune propagation | [70] |
| Permanent MCAO | IV (tail vein) | 7 days | Enhanced brain plasticity, increased synaptophysin, synapse density, and myelinated axons, increased tPA and SHH expression | [71] |
| Permanent MCAO | IV | 24 h | Increased angiogenesis by activation of the Notch signaling pathway | [31] |
| Transient MCAO (90 min) | IC | 8 days | Human MSCs modified cerebral microvasculation, abolished CBV increase and delayed VSI increase | [72] |
| Permanent MCAO | IV | 1 day | Improved functional deficits, increased corticospinal tract axonal density | [73] |
| Transient MCAO (60 min/2 h) | IV (tail vein) | 1/7 days | MSCs reduced infarct volume, expressed cytokines/chemokines/growth factors/neurotropic factors | [74,75] |
| Transient MCAO (1 h) | IC (striatum) | 15 min | BMSCs restored CBF and BBB to normal levels, elevated levels of growth factors/neurotrophic factors | [76] |
| Transient MCAO | IV | 24 h | Migrated towards the scar area and border zone of the ischemic region, expressed the neuronal marker NeuN, reduced scar size, limited apoptosis, enhanced angiogenic factor expression and vascular density, improved functional recovery | [28] |
| Transient MCAO (90 min) | IA | 24 h | MSCs improved functional recovery and reduced the mean infarct volume at the maximum tolerated dose of $1 \times 10^5$ delivered at 24 h | [77] |
| Transient MCAO (90 min) | IA | Immediate | MSCs displayed less reduced amplitude of MEP after monopolar stimulation; 40% of the MSC group had measureable MEP induced by bipolar stimulation and displayed I wave | [78] |

Modified, with permission, from [79].
MCAO: middle cerebral artery occlusion; MCA: middle cerebral artery; SHR-SP: spontaneous hypertension rat stroke-prone; IBZ: ischemic boundary zone; NVU: neurovascular unit; MMP: matrix metalloproteinase; CBF: cerebral blood flow; BMP: bone morphogenetic protein; CX-43: connexin-43; MCP-1: monocyte chemoattractant protein-1; IGF-1: insulin-like growth factor 1; bFGF: basic fibroblast growth factor; BDNF: brain-derived neurotrophic factor; VEGF: vascular endothelial growth factor; GDNF: glial cell line-derived neurotrophic factor; Bcl-2: B-cell lymphoma 2; IL: interleukin; TGF: transforming growth factor; SHH: sonic hedgehog homolog; CBV: cerebral blood volume; VSI: vessel size index; BBB: blood–brain barrier; MEP: motor-evoked potential.

stroke. The rodent model of MCAO involves transecting the external carotid artery, temporarily tying off the common carotid artery, and using the external carotid artery trunk as a side path to pass a suture through the internal carotid artery to lodge in the junction of the anterior cerebral artery and MCA. The suture can be left in place for a variable duration of time and then removed to produce reperfusion. The most common durations of suture occlusion of the MCA in the rat are 60, 90, 120 min, or permanent occlusion. This technique does not require craniotomy, produces focal occlusion of a large cerebral artery, as seen in human stroke, and can be done in a high-throughput manner. The network of cerebral arteries is shown in Figure 48.7, and the area of brain supplied by the MCA is shown in Figure 48.8.

MCAO produces primary ischemic cell death in the striatum and overlying frontal, parietal, temporal, and portions of the occipital cortex, but also variable damage in the thalamus, cervicomedullary junction, substantia nigra, and hypothalamus. The most consistent pattern of cell death in MCAO follows a progression from early infarction in the striatum to delayed infarction in the dorsolateral cortex overlying the striatum. Striatal infarction is mostly necrotic and occurs rapidly. Cortical infarction is more delayed, involves a prolonged and biphasic opening of the BBB, and contains a greater degree of apoptotic cell death than in striatal infarction.

Transplantation of BM-MSCs into striatum has the advantages of requiring lower doses (usually $1–2 \times 10^5$ cells) [39,76] and achieving more rapid effects [50,76]. One study demonstrated that BM-MSCs transplanted into the striatum of rats 15 min after MCAO resulted in recovery of CBF in the infarct area and promoted the recovery of neurological function [76]. Miyamoto *et al.* transplanted BM-MSCs into the striatum 7 days after MCAO using a permanent model of brain damage. Results from $^{18}$F-fluorodeoxyglucose positron emission tomography suggested that BM-MSCs improved the glucose metabolism in the region containing new cortex around the infarct [44]. Li *et al.* found that transplantation of BM-MSCs labeled by

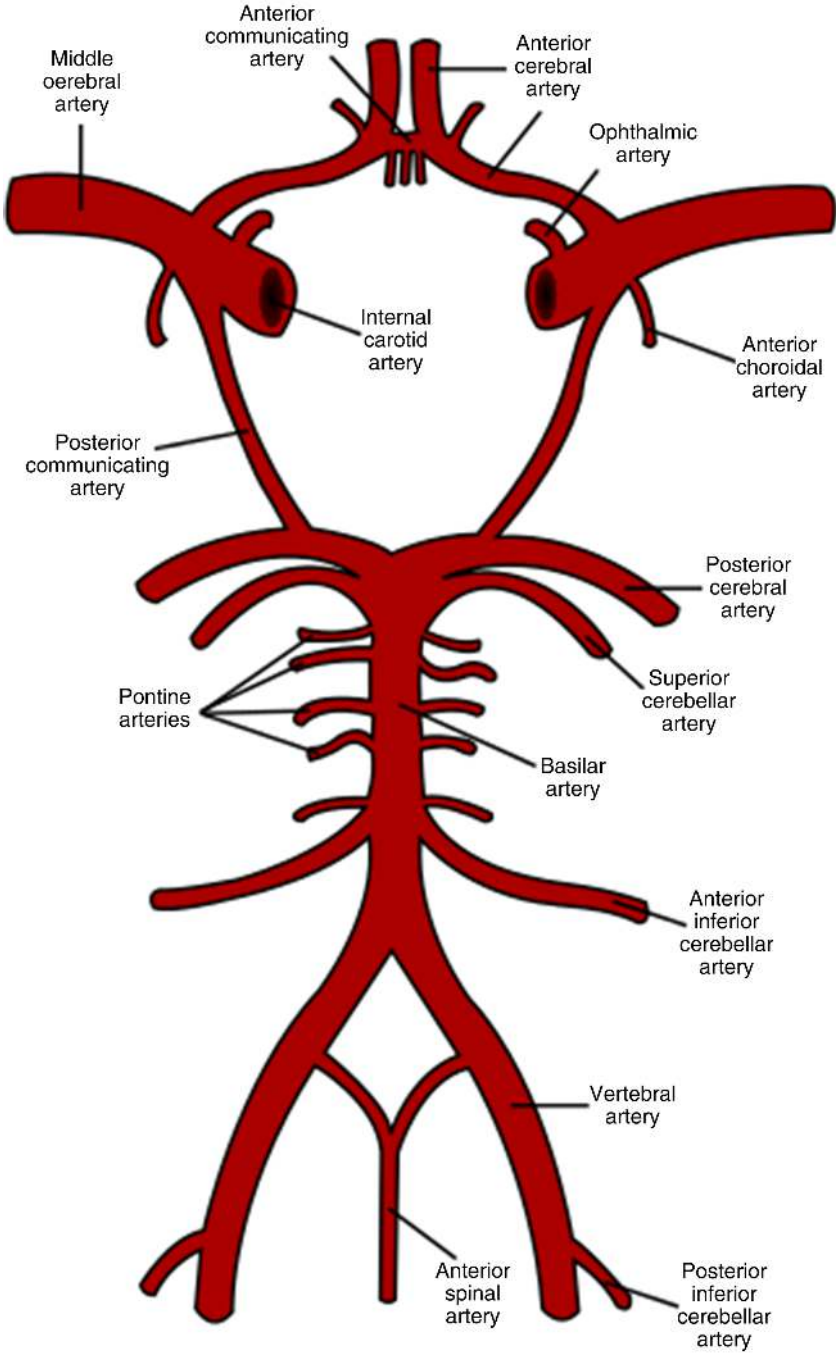



**Figure 48.7** The arteries of the brain. The middle cerebral arteries (top of figure) arise from the internal carotid arteries. *Source:* https://commons.wikimedia.org/wiki/File:Circle_of_Willis_es.svg#/media/File:Circle_of_Willis_es.svg.

bromodeoxyuridine (BrdU) to the striatum of MCAO mice promoted axonal sprouting and remyelination in the ischemic penumbra of the cortex, significantly improved performance on the rotarod test, and modified neurologic severity score tests [39]. (The penumbra is the brain region adjacent to the earliest region of ischemic cell death that will itself progress to infarction over time unless treated.) IC transplantation is relatively invasive and may lead to functional disorders of brain cells. In 14 patients given BM-MSCs into their striatum, seizure, syncopal (fainting) events, or subdural hematoma occurred in three of them [33].

Lateral ventricle injection is another method of IC transplantation in which the transplanted BM-MSCs first adhere to the wall of the lateral ventricle and then penetrate the wall of the ventricle into the ischemic area [37]. Compared with intrastriatal injection, lateral ventricle injection is thought to be less invasive. More BM-MSCs can be transplanted using this method, and the distribution of cells is more extensive [80]. However, the success of lateral ventricle transplantation depends primarily on the properties of the grafted cells, specifically their ability to adapt to CSF and their ability to penetrate the blood–CSF barrier [81].

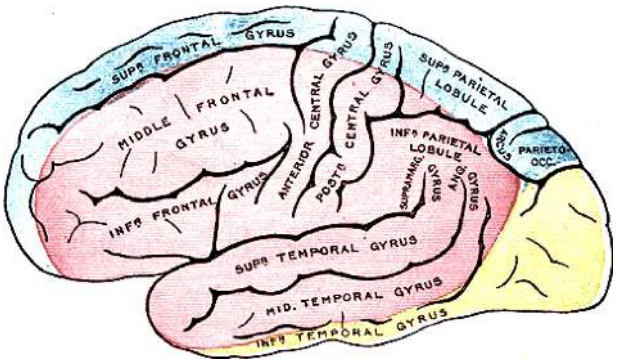



**Figure 48.8** Outer surface of cerebral hemisphere showing areas supplied by the cerebral arteries. The pink region is supplied by the MCA. The yellow region is supplied by the posterior cerebral artery. (See color plate section.) *Source*: this image, reproduced here from Wikimedia Commons, is in the public domain (PD 1923). The author was Henry Vandyke Carter (1831–1897) and the source was Henry Gray (1918) *Anatomy of the Human Body*. Its attribution is as follows: "Gray's Anatomy plate 517 brain" by Henry Vandyke Carter – Henry Gray (1918) *Anatomy of the Human Body* (See "Book" section below) Bartleby.com: Gray's Anatomy, Plate 517. Licensed under Public Domain via Wikimedia Commons – https://commons.wikimedia.org/wiki/File:Gray%27s_Anatomy_plate_517_brain.png#/media/File:Gray%27s_Anatomy_plate_517_brain.png.

The optimum timing and dosage for the transplantation of BM-MSCs via the IC route is not yet clear. Wan *et al.* suggested that the guiding principle behind current treatment approaches for ischemic stroke is "the earlier the better" [82]. Kawabori *et al.* transplanted BM-MSCs into the striatum of MCAO rats at 1 week or 4 weeks after induction of ischemia. Both low-dose and high-dose BM-MSCs significantly improved motor function when BM-MSCs were transplanted at 1 week, whereas only high-dose BM-MSCs improved motor function at 4 weeks. In order to reach the same level of improvement, the number of BM-MSCs transplanted when used early was significantly smaller than the number required at later time points [83].

Increasing evidence supports the conclusion that intravascular transplantation rather than IC transplantation of BM-MSCs is preferable after ischemic stroke [84]. Although IC injection of BM-MSCs is helpful for targeting the transplantation of cells, the therapeutic effect of IV injection is superior for global cerebral ischemia. IV transplantation of BM-MSCs not only allows the cells to distribute more extensively around the lesion [85], but also has the benefits of being a simpler and less invasive procedure and generates cells that can fully interact with various neurotrophic factors and migratory signals [84]. Wu *et al.* suggested that the IV route allowed BM-MSCs to better adapt to an adverse microenvironment [28]. Studies have shown that the use of IV transplantation of BM-MSCs promoted cellular expression of the antiapoptotic proteins Survivin and Bcl-2 and improved sensorimotor function in MCAO rats [57,86]. Transplantation of BM-MSCs via the tail vein promoted the expression of BMP2 and BMP4, gap junction protein CX-43, and synaptophysin [48]. The neurological deficits in rats were significantly alleviated 2 weeks after IV injection and the therapeutic effects of BM-MSCs lasted for at least 1 year [48]. MSCs were intravenously administered at 6 h after a unilateral MCAO in a rat model resulting in bilateral cortical hyperactivity detected by functional MRI and associated with improved motor function [87].

After IV injection the cells become trapped, at least temporarily, in organs such as the lungs and the liver [88]. It is still not clear whether BM-MSCs that localize outside the CNS will cause changes in physiological function in the CNS [79]. Various attempts have been made to improve the therapeutic effects of intravenously transplanted BM-MSCs [28,88]. MSCs have been given in two boluses, blocked with anti-CD49d antibody or infused with multipotent adult progenitor cells, neural stem cells, or bone-marrow-derived mononuclear cells. All of these approaches resulted in an increase in MSCs crossing the pulmonary microvascular barrier [88]. Although the number of BM-MSCs that migrated to the ischemic area was quite small after IV injection, neurological functional deficits improved significantly [84,89]. Goldmacher *et al.* concluded that the functional recovery observed after IV administration of BM-MSCs was likely due to changes in blood and/or brain cell number or their cytokine and growth factor products [90]. Previous studies also indicated that, even with IC administration, the distance of the graft to the lesion may not be important [85,91]. It remains unclear whether enhancing the targeting of BM-MSCs is critical for functional neurological recovery.

IA transplantation of BM-MSCs via the ipsilateral (same side as the ischemic lesion) carotid artery showed a higher survival rate of grafted cells in the ischemic area. This method is easy to use and avoids pterygopalatine artery occlusion [92]. (The pterygopalatine artery is a branch of the maxillary artery, which in turn is the larger of the two terminal branches of the external carotid artery). Compared with the IV route, the IA route is also more suitable for treating transient cerebral ischemia because the transplanted BM-MSCs can travel through the appropriate arteries and reach the ischemic area in a short period of time [48,85]. When the MCAO rat was subjected to 2 h of ischemia followed by 24 h of arterial reperfusion (ischemia–reperfusion (I/R) injury), BM-MSCs transplanted intra-arterially were distributed in the territory of MCA (Figure 48.8), and the transplantated cells led to a significant improvement in neurological deficits and recovery of sensorimotor function [49]. In addition, Ishizaka *et al.* found that IA transplantation provided timing-dependent cell distribution and functional recovery [93]. IA infusion of human MSCs in rats resulted in immediate cell entrapment in the brain, but the majority of the transplanted cells relocated to the internal organs during the next 24 h [94]. Keimpema *et al.* showed that IA injection of a syngeneic suspension of hematopoietic and mesenchymal cells 2 h after MCAO onset resulted in cell migration to the lesion 6 h after transplantation and microglial cells were activated. Despite the difference in the quantities of BM-MSCs detected around the infarct at each time point, the cumulative number of BM-MSCs increased

gradually within 12 h after transplantation and began to decrease at 24 h due to phagocytosis by activated microglial cells. The majority of BM-MSCs (>95%) were eventually trapped in the spleen [51]. Keimpema *et al.* also showed that IA transplantation of BM-MSCs reduced the size of infarct, promoted angiogenesis and neurogenesis, and increased synaptic plasticity by secreting growth factors such as VEGF, bFGF, and BDNF [51]. Jang *et al.* indicated that rats with transient ischemia receiving an IA injection of BM-MSCs displayed less reduced amplitude MEPs after monopolar stimulation. Forty percent of the MSC group had measureable motor-evoked potentials (MEPs) induced by bipolar stimulation and displayed an I wave that was not detected in the control group [78]. Although animal experiments have demonstrated the effectiveness of IA transplantation of BM-MSCs for cerebral ischemia, mortality in the animal model was greatly increased due to arterial microthrombosis [85]. One study showed that the dose of transplanted MSCs was a major determinant of the safety of intracarotid transplantation [95]. This study suggested that an infusion of MSCs (diameter 25 μm) resulted in a significant decrease ($75 \pm 17\%$) in CBF. Stroke lesions occurred frequently (12 out of 15 animals) when injecting $2 \times 10^6$ MSCs, but not after lowering the dose to $1 \times 10^6$ MSCs [95]. Yavagal *et al.* showed that IA delivery of MSCs can be performed safely and effectively at the maximum tolerated dose of $1 \times 10^5$ delivered at 24 h after MCAO [77].

Another delivery method for the transplantation of BM-MSCs is the use of a cell sheet applied to the brain surface [96]. The cell sheet was $27.9 \pm 8.0$ μm thick and contained $9.8 \pm 2.4 \times 10^5$ BM-MSCs. The cells maintained their ability to migrate and to differentiate neurally. This less invasive method of transplantation was also associated with an improvement in neurologic deficits [96].

In the cerebral I/R rat model, the expression of stromal-cell-derived factor-1α (SDF-1α) increased around the ischemic penumbra [97], and chemokine (C–X–C motif) receptor 4 (CXCR4) and chemokine (C–X–C motif) receptor 7 (CXCR7) were expressed mainly by BM-MSCs [98,99]. SDF-1α and CXCR4 played an important role in the migration of BM-MSCs [98], and the effect of SDF-1α/CXCR7 was stronger than that of SDF-1α/CXCR4 [99]. Wang *et al.* showed that treating rats with cerebral ischemia with BM-MSCs led to the expression of SDF-1α, reaching its peak value within 3–7 days and being maintained for at least 14 days after transplantation [100]. Both CXCR4 and CXCR7 bound SDF-1α to synergistically mediate the migration of BM-MSCs to the ischemic area [99,100]. When the expression of CXCR4, CXCR7, or SDF-1α decreased, the number of migrating BM-MSCs decreased correspondingly [99,101,102]. MCP-1 also plays a role in the migration of BM-MSCs. The expression of MCP-1 significantly increased from 6 h and peaked at 48 h after MCAO [52]. In order to evaluate the role of MCP-1, Wang *et al.* added anti-MCP-1 antibody to an extract of the rat ischemic brain tissue and found that the migration of BM-MSCs was reduced [52]. Selectins regulate the distribution of BM-MSCs in the microvascular system after I/R injury. It was considered that cerebral vascular endothelium had a proadhesive role after I/R injury. BM-MSCs adhered to the cerebral vascular endothelium via P- and E-selectin expression by the vasculature and were distributed around the infarct site [103]. CD44, a critical ligand, also participates in selectin-mediated BM-MSC migration. When CD44-deficent BM-MSCs were transplanted into MCAO rats, the targeted migration of BM-MSCs decreased significantly [103].

Other techniques have been described. Migration of BM-MSCs to an ischemic area was improved by using a long course of hyperbaric oxygen treatment [104].

The ultrasound microbubble technique induced the blood-brain barrier (BBB) to open and significantly promoted the migration of MSCs to ischemic brain, leading to an improvement in neurological function of rats [105].

In cerebral ischemia, the infarct tissue eventually becomes necrotic and a cavity forms. This was detrimental to BM-MSC homing [106]. In order to distribute more BM-MSCs around the infarct, an artificial scaffold was transplanted into the ischemic brain [106]. The scaffold was able to encourage new tissues composed of endothelial cells, astrocytes, and microglial cells to grow. Trophic factors produced by these cells promoted BM-MSC migration to the scaffold [107]. One study showed that a fibrin matrix might also improve the survival and migration of BM-MSCs, and therefore be a useful material for injured CNS tissue [108]. A thermoreversible gelation polymer hydrogel could also be a promising candidate for scaffold formation [109].

## 48.3 Tracking techniques for transplanted mesenchymal stromal cells

It is important to develop techniques to serially and non-invasively track the fate of the transplanted cells in the host CNS. Potential tracking technologies of transplanted MSCs include MRI, nuclear imaging, and optical imaging [110].

MRI can be used to observe MSCs labeled by superparamagnetic iron oxide (SPIO) [111–113]. Lu *et al.* showed that *in vivo* MRI was useful for tracking intra-arterially delivered MSCs after SPIO labeling [113]. *In vitro*, high-dose SPIO labeling may affect human MSC survival under serum and oxygen withdrawal conditions [114]. Micrometer-sized SPIOs provided efficient intracellular labeling of human MSCs and did not modify cell viability, phenotype, or *in vitro* differentiation capacity [115]. Detante *et al.* showed that labeled human MSCs can die and release iron particles *in vivo* [115], while Tarulli *et al.* suggested that the use of micrometer-sized SPIOs as labeling agents was insufficient to conclusively determine the localization of iron within cells in regions of ischemia or hemorrhage [116].

Nuclear imaging can detect transplanted cells by labeling them with radioactive tracers [103,117]. Correa *et al.* labeled MSCs with $^{99m}$Tc-hexamethylpropylene and injected them into a patient with an ischemic stroke. The transplanted cells were

visualized using single-photon emission CT [117]. $^{18}$F-fluorodeoxyglucose positron emission tomography may also be a useful tool to visualize the effects of MSC transplantation for ischemic brain lesions [44,118].

The herpes simplex virus thymidine kinase $^{131}$I-5-iodo-2-fluoro-1-β-D-arabinofuranosyluracil (HSV1-TK-$^{131}$I-FIAU) reporter gene-probe system has also been used to track BM-MSC activity in ischemic stroke rat models [119]. Currently, $^{123}$I-iomazenil (IMZ) single-photon emission CT has emerged as a promising technique to track the effects of BM-MSC transplantation on the brain [120].

Optical imaging can also be used to visualize MSCs engrafted in the damaged CNS [29,40,103,121,122]. Shichinohe *et al.* stereotactically transplanted BM-MSCs labeled with green fluorescent protein into the ipsilateral striatum of MCAO mice. Twelve weeks after transplantation the skull was exposed and green fluorescence emitted from the brain surface was sequentially observed using *in vivo* fluorescence optical microscopy [40].

Quantum dots emit near-infrared fluorescence with a longer wavelength (800 nm) and can easily penetrate living tissue. BM-MSCs labeled with quantum dots can be serially visualized by *in vivo* fluorescence imaging for at least 8 weeks after transplantation [121].

Organic nanoparticles with aggregation-induced emission have also been used successfully for tracking MSCs in rats with ischemic stroke, suggesting the potential of fluorescent nanoparticles to help understand the fate of transplanted MSCs [123].

## 48.4 Cytokines and neurotrophic factors

Li and Chopp [124] and others have described MSCs as small molecule factories: they can secrete cytokines, trophic factors, and growth factors. Such factors may be important in the recovery of neurological function after ischemic stroke injury by inducing the migration of BM-MSCs to the ischemic area, providing trophic support, promoting angiogenesis, reducing the apoptosis of neural cells, inducing the differentiation of neural cells, and promoting remodeling of synapses [82].

To induce the migration of BM-MSCs the expression of SDF-1α around the ischemic penumbra and CXCR4 and CXCR7 on the surface of BM-MSCs is upregulated [99,100,125]. Cui *et al.* showed that the molecule nitric oxide donor (DETA NONOate) significantly upregulated CXCR4 and MMPs' expression in BM-MSCs, promoting the migration of BM-MSCs into the periphery of the ischemic area [126]. During the early stage of cerebral infarction, BM-MSCs transplanted into the ischemic area stimulate the expression of many trophic and growth factors, including BDNF, nerve growth factor (NGF), epidermal growth factor, bFGF, IGF-1, hepatocyte growth factor (HGF), VEGF, TGF, angiogenic factor, and stem cell factor [28,29,41,55,82,127–130]. These secreted factors can suppress the local immune system, inhibit fibrosis and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of reparative cells. These effects are referred to as trophic effects and are distinct from the direct differentiation of MSCs for the repair of damaged tissues. Shichinohe *et al.* demonstrated that BDNF may be a key trophic factor for BM-MSCs. Treatment with anti-BDNF antibody significantly reduced the trophic effects of BM-MSCs [41]. Transient receptor potential canonical 6 (TRPC6) is the downstream molecular target of BDNF. The neuroprotective effect of BDNF was significantly suppressed when the TRPC6 channels were blocked or the expression of TRPC6 was reduced [131]. In ischemic stroke, the increased expression of TRPC6 contributed to the protection of neurons [132]. We transplanted MSCs into the striatum of MCAO rats using a 2 h/24 h I/R model. The neurological function of rats was significantly improved. This study suggested that BM-MSCs might increase the expression of BDNF, activate TRPC6 channels, and protect neurons after ischemic stroke. Yilmaz *et al.* showed that angiopoietin (Ang)-1 and -4 were highly expressed in BM-MSCs isolated from post-ischemic brains [133]. The transplantation of human BM-MSCs stimulated the expression of VEGF, which was beneficial for angiogenesis following cerebral ischemia [130]. Sun *et al.* suggested that VEGF also had anti-inflammatatory effects and the ability to promote the migration and differentiation of BM-MSCs in addition to its ability to promote angiogenesis [134–136]. Bcl-2 is a protein capable of inhibiting apoptosis. We transplanted MSCs into the MCAO rat again using the 2 h/24 h I/R model and found that the expression of Bcl-2 protein in areas of cerebral ischemia was increased. Upregulation of Bcl-2 resulted in a decrease of apoptosis [86]. BM-MSCs may upregulate soluble factors, such as BMP2 and BMP4. Both soluble factors play key roles in the differentiation process of astrocytes [48]. It has been shown that the expression of BMP2 and BMP4 is increased through co-culture with BM-MSCs and ischemic astrocytes *in vitro* [137]. Astrocytes express CX-43, which promotes the exchange of small molecules in the brain and improves synaptic function [48]. Intracarotid arterial injection of BM-MSCs improved neurological function and promoted the expression of synaptophysin through increasing the expression of BMP2, BMP4, and CX-43 in astrocytes [48].

Zacharek *et al.* compared the therapeutic effects of BM-MSCs harvested from rats that had stroke-induced cerebral ischemia. The data suggested that BM-MSCs from these rats were superior to those from normal rats, and this may have been due to the enhanced secretion of trophic factors and angiogenic factors by BM-MSCs of rats after a stroke [138]. Hypoxia and reoxygenation pretreatment of human BM-MSCs before their administration increased their ability to rescue ischemic cortical neurons by enhancing trophic factors' release, including VEGF, fibroblast growth factor (FGF) and BDNF [139].

## 48.5 Angiogenesis

Transplantation of BM-MSCs significantly protected NVUs [63] and increased angiogenesis in ischemic brain [31,140]. Ito *et al.*

showed that in stroke-prone spontaneously hypertensive rats (SHR-SP) the transplantation of BM-MSCs protected the neurovascular integrity between basement membrane and astrocyte end-feet, which alleviated the ischemic cerebral injury [63]. Chen *et al.* transplanted human BM-MSCs intravenously into MCAO rats at 24 h after the stroke and showed that the number of new capillaries in the periphery of the infarcts increased significantly [53]. Three-dimensional analysis indicated that the capillary vessel volume in the ischemic region was increased [141]. When MSCs were injected intravenously at 1 month after the MCAO, rats still showed clear improvement of neurological function, suggesting that the systemic delivery of MSCs in the later phase of cerebral ischemia may have beneficial effects through an angiogenic mechanism [142,143].

MSCs may mediate angiogenesis by upregulation of factors such as VEGF, VEGF receptor-2 [53], fetal liver kinase-1, the Ang-1/Tie2 system [144] (the Ang-1 receptor Tie2 is a tyrosine kinase of the Tie family), and erythropoietin (EPO) and EPO receptor (EPOR) [145]. Guo *et al.* transplanted MSCs intravenously at 24 h after MCAO and showed that activation of the Notch signaling pathway was also related to angiogenesis [31]. MSCs [53], MSCs combined with DETA NONOate [144], and human MSCs modified by the Ang-1 gene [146] can all promote functional angiogenesis in the ischemic zone and facilitate recovery of the cerebral blood circulation. The mechanisms by which these cell populations mediated their effect was different. Human BM-MSCs enhanced angiogenesis by increasing the levels of endogenous VEGF and VEGFR2 [53]. Combination treatment of stroke with MSCs and DETA NONOate promoted neovascularization partially by upregulation of the Ang-1/Tie 2 axis [141]. Human MSCs modified by the Ang-1 gene increased angiogenesis at the border of the lesion by hypersecretion of Ang-1 [146].

## 48.6 Neurogenesis

The beneficial effect of MSC administration in stroke animals has been reported to be mediated either by differentiation into neurons and expression of neuronal or glial markers and replacement of dying cells [142,147], or by amplifying endogenous neurogenesis [30].

Li *et al.* transplanted BM-MSCs prelabeled by BrdU into the striatum of MCAO mice and showed that 1% of BrdU-positive cells expressed NeuN, a neuron-specific marker, while 8% of BrdU-positive cells expressed glial fibrillary acid protein (GFAP), an astrocyte-specific protein, at 28 days after the infusion [39]. Schichinohe *et al.* confirmed these findings *in vivo* by tracking transplanted MSCs labeled with green fluorescent protein [40]. MSCs administered by the IC, IV [43], and IA [49] routes in some studies showed similar results. However, Kawabori *et al.* reported that IC, but not IV, administration of bone marrow stromal cells enhanced functional recovery in rat cerebral infarcts [64]. Shichinohe *et al.* transplanted MSCs into the brain after the induction of small deep (lacunar) strokes using a stereotactic technique and suggested that some MSCs expressed neuronal or astrocytic phenotypes in the neocortex, subventricular zone (SVZ), corpus callosum, and perilesional area [59]. Shen *et al.* injected MSCs 1 month after the stroke; subsequent double staining revealed that approximately 13% and 6% of MSCs expressed the astrocyte marker GFAP and the neuronal marker microtubule-associated protein-2 (MAP-2) respectively [142].

Hypoxia pretreatment of MSCs may enhance their ability to differentiate [148]. Wei *et al.* showed that significantly more $NeuN^+$ cells and glucose transporter protein $(Glut\text{-}1)^+$ cells were seen in the ischemic core and peri-infarct regions of animals which received anoxic (0.5%) human MSCs, possibly suggesting that anoxic MSCs differentiated into neuronal and vascular endothelial cells more readily compared with normoxic (20%) MSCs [145]. Rat MSCs transfected with Notch 1 were transplanted into the striatum of rats 1 month after MCAO. Subsequent histological staining showed a 9–15% graft survival rate, with 5% of cells $GFAP^+$ and 23% $NeuN^+$ at 12 weeks after transplantation [149]. When human BM-MSCs transfected with Notch 1 were transplanted into the striatum of rats at 1 month after MCAO, histological analysis revealed a modest 5–7% graft survival rate, and few (<1%) MSCs expressed the MAP-2 neuronal marker [149]. Marrow stromal-cell-derived neuronal cells (MSDNCs) can be specifically induced from BM-MSCs with extremely high efficiency using gene transfection of the Notch intracellular domain and subsequent treatment with bFGF, forskolin, and ciliary neurotrophic factor [150]. Both MSDNCs and BM-MSCs migrated from the injection site into the ischemic boundary area, including the cortex, corpus callosum, striatum, and hippocampus. One month later the survival rates of MSDNCs and BM-MSCs were 45% and 10% respectively [150]; 84% of transplanted MSDNCs were positive for MAP-2 at 41 days after transplantation, whereas only a small number of cells were positive for GFAP [150].

By using a highly hydrophobic diphenylamino-*s*-triazine-bridged *p*-phenylene-coated surface and adopting a procedure for the propagation of neural stem cells, Heo *et al.* efficiently converted MSCs into neurosphere (NS)-like cellular aggregates (NS-MSCs) that were subsequently induced to differentiate into neural cells expressing neuroectodermal markers. NS-MSCs exhibited stronger therapeutic effects than MSCs in rat brains with permanent MCAO [151]. Another study showed that light-depolarization of astrocytes can stimulate the release of endogenous adenosine-5′-triphosphate in rats with cerebral ischemia. The released adenosine-5′-triphosphate acted on transplanted MSCs in the ischemic zone and promoted their differentiation into neurons, with improvement in neurological deficits [152].

The administration of MSCs enhanced endogenous neurogenesis in the SVZ [48,54,59,127], which is one of the sites in the brain where endogenous neural stem cells develop. Zhang *et al.* transplanted human BM-MSCs via the IA route 24 h after an ischemic stroke and found that there were more $BrdU^+$ and $Ki\text{-}67^+$ cells in the SVZ, as well as more $BrdU^+/GFAP^+$ cells in the

IBZ [48]. Similarly, Li *et al.* and Chen *et al.* showed enhanced neurogenesis in the SVZ by transplanting either human BM-MSCs or human BM-MSCs with DETA NONOate [54,127].

## 48.7 Axonal sprouting and remyelination

Studies have shown that, after stroke, transplanted MSCs can facilitate the recovery of neural function, enhance interhemisphere and intercortical connections, and restore connections between different cerebral areas by axonal sprouting and remyelination of surviving cortical neurons around the IBZ [67,153]. Axonal sprouting and remyelination may be responsible for the neurological functional improvement observed after MSC administration [50]. Li *et al.* transplanted allogeneic and syngeneic MSCs intravenously into rats 24 h after ischemia and found significant increases in the thickness of axons and myelin, areas of the contralateral corpus callosum, and the number of white matter bundles in the striatum [154]. Song *et al.* provided novel evidence that the transplantation of MSCs has the potential to repair ischemia-damaged neural networks and restore lost neuronal connections. The expression of the axonal growth-associated protein-43 was significantly increased, whereas expression of the axonal growth inhibiting proteins Rho-associated protein kinase II and neural/glial antigen 2 were suppressed in MSCs-treated brains [61].

Ding *et al.* showed that MSCs significantly enhanced brain plasticity after ischemia, and also led to increases in synaptophysin, synapse density, myelinated axons along the IBZ, and endogenous tPA and SHH expression [71]. tPA plays a pivotal role in neurite outgrowth via the SHH signaling pathway [71]. (A neurite refers to any projection from the cell body of a neuron. This projection can be either an axon or a dendrite). Co-culture of MSCs with oxygen–glucose-deprived cortical neurons confirmed the effect of SHH on MSC-mediated neurite outgrowth [71]. Plasminogen activator inhibitor 1, the inhibitor of tPA, also participates in regulating neurite outgrowth [68]. Xin *et al.* showed that MSCs decreased TGF-β1 expression in the IBZ, which contributed to downregulation of plasminogen activator inhibitor 1, thus inducing neurite outgrowth after stroke [68].

Cultured neurons treated with exosome-enriched fractions from MSC-exposed rat brain extracts 72 h after MCAO showed significantly increased neurite branch number and total neurite length [155]. Futher, Xin *et al.* demonstrated that the therapeutic benefit of MSC treatment in ischemic stroke is mediated by miR-133b via exosomes to neural cells that improve neurite remodeling and brain plasticity [156].

## 48.8 Antiapoptotic effects

One of the benefits of MSC administration after stroke injury is a reduction in apoptosis in the penumbral zone of the ischemic lesion. Apoptosis is the process of programmed cell death that occurs in multicellular organisms. Biochemical events lead to characteristic cell changes and death. These changes include blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, chromosomal DNA fragmentation, and global mRNA decay. A diagram of the process of apoptosis is shown in Figure 48.9.

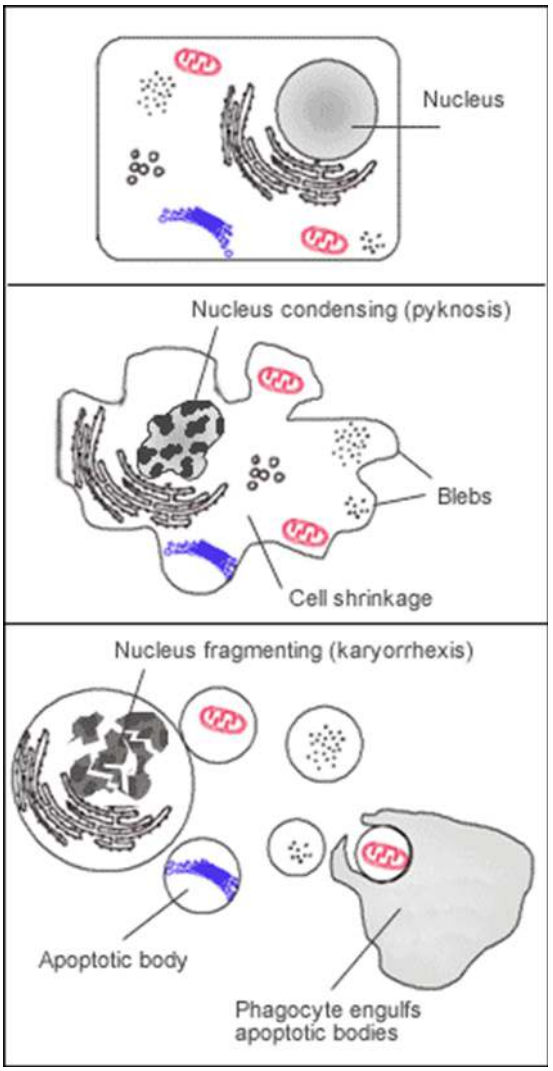



**Figure 48.9** Labeled diagram of a cell undergoing apoptosis. *Source:* https://commons.wikimedia.org/wiki/File:Apoptosis.png#/media/File:Apoptosis.png/

Several studies have shown that IV administration of MSCs to MCAO rats reduced apoptosis along the IBZ [28,55,66,157]. MSCs protected the integrity of the BBB by reducing apoptosis of astrocytes after ischemic stroke [158]. Calió *et al.* suggested that the transplantation of MSCs decreases apoptosis in the brain of SHR-SPs by increasing expression of the antiapoptotic gene *Bcl-2* [159]. In our study, upregulation of *Bcl-2* resulted in a decrease of apoptosis, and may be one of the therapeutic mechanisms by which MSCs mediate a benefit in cerebral ischemic stroke injury [86].

The *in vitro* proliferation of neuronal cells was significantly decreased, and the rate of apoptosis increased, after exposure to oxygen–glucose deprivation. These effects could be alleviated by co-culture with MSCs which had the capacity to decrease apoptosis [160]. Scheibe *et al.* investigated the mechanism through which MSCs protect neurons from apoptosis in oxygen–glucose deprivation. They showed that MSCs activate signal transducer and activator of transcription-3- and Akt-dependent antiapoptotic pathways that enhance the survival of neurons after oxygen–glucose deprivation [161].

Genetically modified MSCs showed greater potential of an antiapoptotic effect. Kurozumi *et al.* used a fiber-mutant F/RGD adenovirus vector to transfect the BDNF gene to human MSCs (BDNF-hMSCs) [162]. Rats receiving BDNF-hMSCs exhibited greater functional recovery, and the number of terminal deoxynucleotidyl transferase dUTP nick-end labeling-positive apoptotic cells in the IBZ was significantly decreased [162]. Zhao *et al.* suggested that MSCs transfected with HGF also had an antiapoptotic effect [163].

## 48.9 Immunomodulation

Another mechanism by which MSCs may mediate a beneficial effect in stroke injury is by their immunoregulatory effects. Xenotransplantation of MSCs is feasible since MSCs are relatively nonimmunogenic [42]. In the early stage of ischemic stroke, cell death results in a release of antigens. Oxidative stress and inflammation mediators activate T cells that cross the BBB [164]. T cells probably exert detrimental effects by antigen recognition [165]. The infiltrating T cells generate large quantities of interferon-γ (IFN-γ), which mediates delayed neurotoxicity in ischemic areas [165]. Transplantation of MSCs inhibits T cell proliferation and cytotoxicity and reduces IFN-γ production by hindering the T cell response to cognate peptides [166]. MSCs also significantly reduce the infiltration of gamma delta T cells, which are detrimental to the ischemic brain, and increase levels of regulatory T cells (Tregs) and Treg-associated cytokines in the ischemic brain [167]. IL-10 induces the protective effect of Tregs by limiting the activation of proinflammatory T cells and decreasing inflammatory cytokines such as IFN-γ [168]. Liu *et al.* suggested that MSCs could increase the expression of IL-10 and decrease tumor necrosis factor-α and thus limit ischemic brain injury [46]. IL-23/IL-17 induce inflammation in cerebral ischemia [60]. MSCs play an immunomodulatory role by reducing IL-23/IL-17 levels, both in serum and around the infarct lesion [60]. Yoo *et al.* demonstrated that the transplantation of MSCs enhanced the recovery of brain functions by secreting TGF-β1, which could block the upregulation of MCP-1 and reduce the infiltration of $CD68^+$ cells and thus limit immune propagation in the ischemic rat brain [70].

However, levels of the inflammatory cytokines IL-6, tumor necrosis factor-α, IFN-γ, and MCP-1 remained unchanged in the sera of mice after cerebral ischemia and transplantation of MSCs [169].

No negative effects of MSCs transplantation on the immune system after stroke have been observed, which may reduce safety concerns about the administration of MSCs in ongoing clinical trials in people who have had an ischemic stroke [169].

## 48.10 Pretreatment of mesenchymal stromal cells prior to their administration in animal models of ischemic stroke

In animals with cerebral ischemia, transplanted MSCs migrate to the ischemic area [28], express trophic factors [29], increase angiogenesis [31], promote neurogenesis [30], improve synaptic plasticity [67], and inhibit apoptosis [66]. To achieve a better treatment effect, different ways of pretreating the MSCs prior to their administration in animal models of ischemic stroke have been explored [26,79] (Table 48.2).

**Table 48.2** Pretreatment of MSCs prior to their administration in animal models of ischemic stroke.

| Stroke model | Cell delivery route | Cells administered | Timing | Results | References |
|---|---|---|---|---|---|
| Transient MCAO (2 h) | IC | FGF-2/HGF–MSCs | 2 h/24 h | Remarkable decrease of infarct volume, neuroprotective or vasodilating effect of FGF-2 and HGF | [163,170] |
| Permanent MCAO | IC | G-CSF–MSCs | 7 days | Upregulated expression of SDF-1α, HGF, and NGF | [129] |
| Permanent MCAO | IV | BDNF/GDNF/PLGF/VEGF–human MSCs (modified to hypersecrete neurotropic factors) | 6 h/3 h | Decreased infarct size and promoted functional recovery | [146,171–173] |
| Permanent MCAO | IC | MSDNCs–bFGF/CNF | 7 days | Behavioral functional recovery in beam balance test, limb placing test, and Morris water maze tests | [150] |

**Table 48.2** (*Continued*)

| Stroke model | Cell delivery route | Cells administered | Timing | Results | References |
|---|---|---|---|---|---|
| Barrel cortex ischemia | IC | Hypoxic MSCs | 24 h | MSCs decreased infarct size and restored thalamocortical circuit connections | [61,174] |
| Focal cerebral ischemia | Intra-nasal | Hypoxic MSCs | 24 h | MSCs decreased cell death in IBZ and reduced infarct volume. Hypoxic preconditioning enhanced cell homing and optimized therapeutic efficacy | [175] |
| Permanent MCAO | IV | Noggin–MSCs | 5 days | Promoted neurogenesis and decreased infarct volume, and ameliorated neurological deficits | [176] |
| Permanent MCAO | IV | CXCR4–MSCs | 24 h | Promoted mobilization and migration, enhanced neuroprotection | [98] |
| Permanent MCAO | IV | NGF/Noggin–MSCs | 5 days | Improved neurological function, upregulated neuron-like cell differentiation and increased synaptophysin expression | [177] |
| Permanent MCAO | IC striatum | PL/G-CSF–human MSCs | 7 days | Enhanced proliferation | [178] |
| Transient MCAO | IV | BDNF–MSCs | 48 h | Improved neurological deficits, reduced infarct volume, improved BBB dysfunction, inhibited neuronal apoptosis | [179] |
| Transient MCAO | IV | VEGF–MSCs | 1 day | Promoted angiogenesis and increased CBF in the ischemic areas, improved neurological deficits | [180] |
| Transient MCAO (2 h) | IV | MCI-186–MSCs | Immediate | Upregulated secretion of BDNF, VEGF, superoxide dismutase; inhibited apoptosis; improved functional deficits; reduced infarct volume; increased the number of neurons | [181] |
| Transient MCAO (90 min) | IV | Hypoxic MSCs | 24 h | Enhanced survival of transplanted cells, improved regenerative properties, upregulated trophic/growth factors, downregulated proinflammatory cytokines and chemokines, expressed more $NeuN^+$ cells | [145] |
| Transient MCAO (1 h) | IV | VPA/lithium–MSCs | 24 h | Improved functional recovery, reduced brain infarct volume, enhanced angiogenesis, migrated toward IBZ | [182] |
| Transient MCAO (2 h) | IV | MSCs | 24 h | Improved transplanted cells' survival capacity and upregulated the expression of protective cytokines | [183] |
| Transient MCAO (2 h) | IC | FlK-1–human SCs | 3 days | Exhibited significant behavioral recovery, increased glucose metabolic activity and reduced infarct volume, increased expression of VEGF and BDNF, promoted angiogenesis, facilitated CBF | [184] |
| Transient MCAO (2 h) | IC | EPO–MSCs | 14 days | Improved neurological function and reduced infarct volume, secreted various trophic factors | [185] |
| Permanent MCAO | IC striatum | NS-MSCs | 3 days | Highly distributed throughout the area of lesion; expressed dopaminergic, glutaminergic and GABA neuronal makers, extended neurites, expressed synaptophysin, improved functional recovery | [186] |
| Permanent MCAO | IV | Ang–VEGF–human MSCs | 6 h | Reduced infarct volume, increased angiogenesis, improved functional recovery | [187] |
| Transient MCAO 2 h | IV | HIF 1α–MSCs | 6 h | Significantly improved motor function, reduced infarct volume and increased VEGF expression | [188] |

Modified, with permission, from [79].
G-CSF: granulocyte-colony stimulating factor; PLGF: placental growth factor; CNF: ciliary neurotrophic factor; CXCR4: chemokine (C–X–C motif) receptor 4; VPA: valproic acid; HIF-1α: hypoxia inducible factor-1α; GABA: γ-aminobutyric acid; PL: platelet lysate.

### 48.10.1 Administration of genetically engineered mesenchymal stromal cells

The IV injection of human MSCs transfected with genes of various growth and neurotrophic factors significantly improved neurological function in ischemic stroke. These factors include BDNF, GDNF, FGF, VEGF, and PLGF [170–173,180]. Ikeda *et al.* showed that intrastriatal delivery of MSCs transfected with FGF-2 improved neurological function and reduced infarct volume in MCAO rats [170]. This improvement was mainly attributed to the transfected FGF-2 playing a neuroprotective or vasodilatory effect [170]. Human MSCs transfected with PLGF were transplanted intravenously into MCAO rats and showed greater therapeutic effect [173]. PLGF promoted neuroprotection and angiogenesis in cerebral ischemia [173]. The modified MSCs were able to overexpress corresponding growth and neurotrophic factors and had a greater effect in reducing infarct volume and improving neurological function [170–173,180]. A positive correlation between the expression levels of modified genes and the therapeutic effect of modified MSCs was found [172,173]. Factors such as Ang-1, Ang-2, and VEGF play important roles in angiogenesis [146,180,187]. MSCs transfected with the gene for VEGF upregulated the level of endogenous Ang-2, which promoted angiogenesis in MCAO rats [180]. The IV administration of human MSCs transfected with the genes for Ang-1 and VEGF (Ang-1–VEGF–human MSCs) showed greater structural–functional recovery than Ang–human MSCs or VEGF–human MSCs [187].

HIF-1α plays a stimulatory role in revascularization in cerebral ischemia. MSCs transfected with a HIF-1α mutant gene were transplanted into MCAO rats and showed significantly improved motor function, reduced infarct volume, and increased VEGF protein expression [188]. The interaction between SDF-1α (CXCL12) and its cognate receptor CXCR4 is crucial for BM-MSC homing and migration [97,98]. MSCs transfected with the gene for CXCR4 promoted the migration of MSCs to the ischemic zone and enhanced neuroprotective effects, which contributed to increasing capillary vascular volume of the peri-infarct area, reduced infarct volume, and improved neurological function in MCAO rats [98].

Hypoxic pretreatment of MSCs before transplantation improved the survival rate of the grafted cells [145]. Hypoxia exposure upregulated endogenous HIF-1α and neurotrophic and growth factors in MSCs, including BDNF, GDNF, VEGF, EPO and EPOR, SDF-1, and CXCR4, and reduced the secretion of many proinflammatory cytokines and chemokines [145]. The transplantation of human MSCs in MCAO rats resulted in better locomotion recovery in the rotarod test [145].

The barrel cortex ischemic stroke model in rodents is generally used to evaluate the recovery of damaged neuronal loop connectivity [174]. The cortical representation of each facial whisker in a rodent forms a structural module, or barrel, that is easily visualized in the adult brain. Together, these modules constitute the barrel cortex, which forms a significant part of the entire rodent cortex. To generate reproducible stroke and a component of reperfusion injury, arterial branches to the barrel cortex are permanently interrupted and both common carotids are occluded for 1 h. The outcome of this technique is a small cortical stroke in a specific functionally defined region of the rat barrel field. Human MSCs (cultured for 24 h in 0.5% $O_2$) transplanted to adult mice 1 and 7 days after focal barrel cortex stroke promoted angiogenesis and axonal growth [61]. Six weeks after the stroke, the ischemia-damaged intracortical activity showed noticeable recovery and the thalamocortical connections were partially restored [61]. Kim *et al.* administered autologous MSCs intravenously in patients with ischemic stroke. The autologous MSCs were cultured *in vitro* with autologous ischemic serum obtained at the time of the acute phase of the stroke [189]. Tsai *et al.* showed that there was no difference in the effectiveness of MSC conditioned medium (CM) derived from ischemic or normal animals [190]. The IV infusion of MSCs grown in normal CM and those grown in ischemia CM were both associated with improved functional recovery, increased neurogenesis, and attenuated microglia and macrophage infiltration in MCAO rat brains [190]. *In vitro*, both populations of MSCs effectively increased neuronal connections and enhanced survival in mixed neuron–glial cultures [190].

Noggin, an antagonist of BMP, regulates the differentiation of stem cells into neurons [191]. BM-MSCs transfected with Noggin and then injected intravenously into MCAO rats promoted neurogenesis in the ipsilateral SVZ, enhanced neuroprotective effects, reduced infarct volume, and improved functional deficits [176]. The cotransfection of NGF and Noggin in BM-MSCs induced synergistic effects on functional recovery and brain plasticity after ischemic stroke [177].

Previous studies have confirmed the therapeutic effects of the transplantation of BM-MSCs on cerebral ischemia. However, the proliferative, differentiation, and homing capacities of BM-MSCs from an elderly population are significantly reduced, especially after several rounds of expansion *in vitro*. By introducing lentivirus-mediated human telomerase reverse transcriptase and VEGF genes into human BM-MSCs from aged donors, Tang *et al.* observed extended lifespan and increased angiogenic capacity [192].

Thus, different pretreatment regimens of BM-MSCs can promote the migration of MSCs toward the ischemic area, provide trophic support to ischemic brain, increase functional angiogenesis, and induce neurogenesis.

### 48.10.2 Administration of mesenchymal stromal cells in combination with chemical agents

Several studies have demonstrated that the application of a number of agents can induce BM-MSCs to migrate to the ischemic area, modulate the expression of cytokines and trophic factors, promote angiogenesis, inhibit apoptosis of the transplanted BM-MSCs, and enhance their therapeutic effect [109,125,144,193].

Intracranial transplantation of BM-MSCs in combination with SDF-1α promotes the migration of BM-MSCs to the ischemic region, increases the density of blood vessels in the ischemic cortex, enhances the plasticity of neurons, and upregulates the expression of antiapoptosis proteins [102].

MCI-186 (3-methyl-1-phenyl-2-pyrazolin-5-one) is a free-radical scavenger that enhances NGF protein levels via the c-Jun NH2-terminal kinase pathway. When BM-MSCs combined with MCI-186 were transplanted, MCI-186 enhanced the survival of the BM-MSCs, promoted the migration of BM-MSCs to the ischemic area, and promoted neurogenesis around the ischemic penumbra [181].

Osanai *et al.* grafted a BM-MSC–thermoreversible gelation polymer (TGP) hydrogel construct on to the ipsilateral intact neocortex of adult mice 7 days after MCAO [109]. The TGP hydrogel promoted the migration of BM-MSCs to the cerebral infarct zone as well as the adjacent dorsal neocortex. TGP hydrogel is biodegradable and provoked no inflammation in the host brain [109].

The IV infusion of human MSCs with DETA NONOate significantly improved functional recovery in transient (2 h) MCAO rats [54]. After this combination therapy, cell proliferation in the SVZ and the expression of VEGF and bFGF in the IBZ increased [54]. DETA NONOate induced high expression of CXCR4 and MMP in BM-MSCs, which promoted their migration and adhesion to ischemic brain [126]. The combination of BM-MSCs and DETA NONOate also increased the expression of Ang-1 and its receptor Tie2, prompted angiogenesis, and restored cerebral blood circulation [144].

Sodium ferulate, the sodium salt of ferulic acid, is a compound used in traditional Chinese medicine for the treatment of cardiovascular and cerebrovascular diseases and to prevent thrombosis. It is considered safe and effective. The coadministration of BM-MSCs and sodium ferulate promoted the expression of SDF-1α/CXCR4 and improved the homing of BM-MSCs toward the ischemic zone [125]. This study showed that serum ferulate induced the differentiation of BM-MSCs to Nestin$^+$ cells, promoted the migration of Nestin$^+$ cells to the ischemic area, and improved functional recovery [125]. Zhao *et al.* also suggested that the administration of BM-MSCs and sodium ferulate facilitated the differentiation of BM-MSCs into neural- and astrocyte-like cells, as well as enhancing the repair capacity of cerebral parenchymal cells by promoting glucose metabolism and endogenous neurogenesis after stroke [194].

*Z*-Val-Ala-DL-Asp-FMK (Z-VAD) is a caspase family inhibitor and cell permeable apoptotic protease. When BM-MSCs were transplanted in combination with Z-VAD into I/R MCAO rats, the survival rate of BM-MSCs significantly increased while the apoptotic rate decreased [193].

Massive intracerebral hemorrhages were observed in the ischemic hemispheres with tPA treatment after MCAO. Edaravone is a nootropic and neuroprotective agent used for the purpose of aiding neurological recovery following acute brain ischemia and subsequent cerebral infarction. Nootropic is a term given to memory enhancers, neurological enhancers, cognitive enhancers, and intelligence enhancers. Nootropics are drugs, supplements, nutraceuticals, and functional foods that improve one or more aspects of mental function, such as working memory, motivation and attention. They act as a potent antioxidant and strongly scavenge free radicals, protecting against oxidative stress and neuronal apoptosis. The combination of BM-MSCs and edaravone protected the ischemic brain against the risk of tPA. Only small hemorrhages were found in recipients of BM-MSCs and edaravone [195].

Oxiracetam is a nootropic drug of the racetam family. The proven effects of the drug are limited to beneficial effects that lead to higher scores in tests for logical performance, attention, concentration, memory, and spatial orientation. Oxiracetam has a protective effect in cerebrovascular impairment [196]. The coadministration of BM-MSCs and oxiracetam showed a synergic effect in improving neurologic function, increasing the expression of Bcl-2, and inhibiting apoptosis in MCAO rats [86].

Minocycline is a broad-spectrum antibiotic that is also a neuroprotective agent and may lead to greater histological and functional improvement when combined with BM-MSCs [197]. Statins, a commonly prescribed type of medication used to lower cholesterol levels, also prevent thrombosis and arteriosclerosis and protect endothelium [198]. The combination of BM-MSCs and simvastatin improved neurological function primarily due to the pharmacological effects of simvastatin that reduce infarct volume and alleviate brain edema [198]. Recent studies showed that BM-MSCs in combination with Chinese medicines, including Bing Pian (Borneolum), She Xiang (Moschus), Naomai Tong, and Dihuang Yinzi, promoted the homing of BM-MSCs, induced angiogenesis, and removed blood clots in ischemic stroke [199,200].

Balseanu *et al.* tested the hypothesis that administration of BM-MSCs in G-CSF-treated animals improved long-term functional outcome in aged rodents. However, the studies showed that, in post-stroke aged rats, daily treatment with G-CSF alone led to a robust and consistent improvement of neurological function. The combination of BM-MSCs with G-CSF may not have any impact on the spatial reference–memory task or infarct volume and did not further improve recovery after stroke injury [201].

## 48.11 Clinical trials involving bone-marrow-derived mesenchymal stromal cells in the treatment of ischemic stroke

The transplantation of BM-MSCs has had a useful therapeutic effect on cerebral ischemia in animal models [138]. In 2005, Bang *et al.* administered autologous MSCs intravenously to five patients with ischemic stroke [202]. One year after transplantation the patients receiving MSCs showed a lower modified Rankin scale score corresponding with improvement in

**Table 48.3** Classification severity of stroke.

| Score | Stroke severity |
|---|---|
| 0 | No stroke symptoms |
| 0–4 | Minor stroke |
| 5–15 | Moderate stroke |
| 16–20 | Moderate to severe stroke |
| 21–42 | Severe stroke |

neuroimaging. No side effects from the MSC infusions were detected [202]. This study suggested that IV administration of autologous MSCs for ischemic stroke is safe. However, because the number of patients receiving MSC treatment was small, Lee *et al.* administered autologous MSCs intravenously to 16 patients with ischemic stroke and followed them up for as long as 5 years [34]. The mortality rate during the follow-up period decreased in the MSC group. Again, it was found that IV MSC administration was safe in stroke patients [34]. Friedrich *et al.* administrated autologous MSCs intra-arterially to patients who had had an ischemic stroke within the preceding 3–7 days [203]. Serial clinical, laboratory, electroencephalogram, and imaging evaluations showed no procedure-related adverse events. The USA National Institutes of Health Stroke Scale (NIHSS) is a tool used by health-care providers to objectively quantify the impairment caused by a stroke (Table 48.3).

Lower NIHSS scores occurred in 6/20 (30%) patients at 90 days after the stroke [203]. Moniche *et al.* administrated autologous MSCs intra-arterially to patients who had an ischemic stroke within the preceding 5–9 days and reported improved neurological function [36]. Battistella *et al.* administrated autologous MSCs intra-arterially to patients with an MCA ischemic stroke within 90 days of symptom onset [35]. No patients exhibited any adverse events during the procedure. There was no worsening in the neurological scales during the follow-up period [35]. These studies indicate that transplantation of MSCs for an ischemic stroke is safe regardless of the time of administration of the MSCs [35,36,203].

In the majority of animal experiments BM-MSCs have generally been transplanted 24 h to 7 days after the stroke [44,45], whereas in clinical trials the timing has usually been several weeks to several months after the stroke [34,35,202,204]. Changes are required in the way clinical trials for ischemic stroke are carried out [205]. Importantly, larger numbers of patients should be recruited to both further phase I and to phase II trials.

G-CSF may be useful for accelerating BM-MSC expansion in order to shorten the interval between the stroke and the administration of MSCs [129,178]. An appropriate concentration of G-CSF significantly enhances BM-MSC proliferation by modulating their cell cycle and also upregulates production of NGF, HGF, and SDF-1α [129,178]. One study showed that G-CSF activated the proliferative capacity of BM-MSCs in aged animals [206]. The Quantum cell expansion system is a novel method of generating high numbers of MSCs in a shorter period of time and requires fewer passages than current conventional methods [207].

## 48.12 Controversies and safety analysis of bone-marrow-derived mesenchymal stromal cells in the treatment of ischemic stroke

### 48.12.1 Conflicting results in animal models

Whether transplantation of BM-MSCs into the ischemic brain can cause adverse effects remains controversial in spite of the numerous animal models and the early clinical trials described previously. Chen *et al.* showed that the administration of BM-MSCs in a type 1 diabetic MCAO (T1DM-MCAO) rat model did not improve neurological deficits but rather resulted in leakage of the BBB and promoted the occurrence of arteriosclerosis [208]. However, Ye *et al.* showed that the transplantation of BM-MSCs combined with extended-release nicotinic acid (Niaspan), also known as vitamin B3, to T1DM-MCAO rats improved white matter remodeling, promoted myelination, and enhanced synaptic plasticity [209]. Yan *et al.* suggested that BM-MSCs with Niaspan may alleviate leakage of the BBB and arteriosclerosis-like changes by decreasing the expression of angiogenin, MMP9, and ED1 (a cellular marker specific for activated rat microglia, monocytes, and macrophages) in T1DM-MCAO rats [210]. Mitkari *et al.* showed that BM-MSCs, although they could migrate to the ischemic penumbra and promote angiogenesis around the lesion, could not improve neurological function of rats after an ischemic stroke [69]. Steiner *et al.* found that IV administration of human BM-MSCs in mice at 24 h after MCAO did not result in any significant differences in mortality between the treatment group and the control group [211]. The majority of the MSCs localized in lung, spleen, kidney, and intestine but not in the brain [211]. Boltze *et al.* observed that, although many studies have indicated that the delivery of MSCs in stroke improves functional outcomes, commonly applied functional tests may not be suitable for discriminating real functional recovery from compensation, which is a frequently encountered phenomenon in rodents. Consequently, these workers highlighted some alternative tests that might determine specific functions, including the cylinder task, tapered/ledged beam walking, and Montoya's staircase test, all of which are relatively easy to perform [212].

### 48.12.2 Combined transplantation of mesenchymal stromal cells and neural stem/precursor cells

Eight patients with cerebral ischemia were enrolled in a clinical trial to assess the safety and feasibility of the cotransplantation of neural stem/precursor cells and MSCs [213]. The results indicated that the most common side effect of cell

transplantation was low fever, which usually lasted for 2–4 days after each administration of cells. All side effects appeared within the first 2–24 h after cell transplantation and resolved spontaneously. There was no evidence of neurological deterioration or infection, and no tumor formation was found at 2-year follow-up. These observations support the use of the combination transplantation of neural stem/precursor cells and MSCs as a safe and feasible method of improving neurological function [213]. Lalu *et al.* performed a systematic review of the literature reporting on over 1000 patients who had received MSCs by either the IV or the IA route. They did not identify any adverse events other than transient fever [214].

## 48.13 Conclusions and perspectives

BM-MSCs are remarkable cells with potential for treating a wide variety of human diseases, including ischemic stroke. They have the ability to improve repair of cerebral ischemic damage by neuroprotection, angiogenesis, neurogenesis, axonal sprouting and myelin remodeling, antiapoptosis, and immunoregulation. A large number of preclinical studies have consistently shown that BM-MSCs are safe and improve neurological function after stroke. Initial human trials have also indicated that they are safe. Much further preclinical work remains to be done, but preclinical animal models and early clinical trials support the value of administration of BM-MSCs as a treatment for ischemic stroke.

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## References

1 S. C. Johnston, S. Mendis, C. D. Mathers. Global variation in stroke burden and mortality: estimates from monitoring, surveillance, and modelling. *Lancet Neurol* 2009;**8**:345–354.
2 G. A. Donnan, M. Fisher, M. Macleod, S. M. Davis. Stroke. *Lancet* 2008;**371**:1612–1623.
3 R. W. Teasell. Long-term sequelae of stroke: how should you handle stroke complications? *Can Fam Physician* 1992;**38**:381–388.
4 S. Ansari, M. Rahman, M. F. Waters, *et al.* Recanalization therapy for acute ischemic stroke, part 1: surgical embolectomy and chemical thrombolysis. *Neurosurg Rev* 2011;**34**:1–9.
5 W. Hacke, M. Kaste, E. Bluhmki, *et al.* Thrombolysis with alteplase 3 to 4.5 hours after acute ischemic stroke. *N Engl J Med* 2008;**359**:1317–1329.
6 O. Adeoye, R. Hornung, P. Khatri, D. Kleindorfer. Recombinant tissue-type plasminogen activator use for ischemic stroke in the United States: a doubling of treatment rates over the course of 5 years. *Stroke* 2011;**42**:1952–1955.
7 J. S. Dubow, E. Salamon, E. Greenberg, A. Patsalides. Mechanism of acute ischemic stroke in patients with severe middle cerebral artery atherosclerotic disease. *J Stroke Cerebrovasc Dis* 2014;**23**:1191–1194.
8 Y. C. Chang, W. C. Shyu, S. Z. Lin, H. Li. Regenerative therapy for stroke. *Cell Transplant* 2007;**16**:171–181.
9 A. Jablonska, B. Lukomska. Stroke induced brain changes: implications for stem cell transplantation. *Acta Neurobiol Exp (Wars)* 2011;**71**:74–85.
10 T. Bliss, R. Guzman, M. Daadi, G. K. Steinberg. Cell transplantation therapy for stroke. *Stroke* 2007;**38**:817–826.
11 S. Law, S. Chaudhuri. Mesenchymal stem cell and regenerative medicine: regeneration versus immunomodulatory challenges. *Am J Stem Cells* 2013;**2**:22–38.
12 D. S. Krause, N. D. Theise, M. I. Collector, *et al.* Multi-organ, multilineage engraftment by a single bone marrow-derived stem cell. *Cell* 2001;**105**:369–377.
13 E. L. Herzog, L. Chai, D. S. Krause. Plasticity of marrow-derived stem cells. *Blood* 2003;**102**:3483–3493.
14 E. Drela, K. Stankowska, A. Kulwas, D. Rosc. Endothelial progenitor cells in diabetic foot syndrome. *Adv Clin Exp Med* 2012;**21**:249–254.
15 M. Witkowska-Zimny, E. Wrobel. Perinatal sources of mesenchymal stem cells: Wharton's jelly, amnion and chorion. *Cell Mol Biol Lett* 2011;**16**:493–514.
16 J. S. Mohal, H. D. Tailor, W. S. Khan. Sources of adult mesenchymal stem cells and their applicability for musculoskeletal applications. *Curr Stem Cell Res Ther* 2012;**7**:103–109.
17 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.
18 S. Zhou. From bone to brain: human skeletal stem cell therapy for stroke. *Cent Nerv Syst Agents Med Chem* 2011;**11**:157–163.
19 E. H. Javazon, K. J. Beggs, A. W. Flake. Mesenchymal stem cells: paradoxes of passaging. *Exp Hematol* 2004;**32**:414–425.
20 S. E. Haynesworth, M. A. Baber, A. I. Caplan. Cell surface antigens on human marrow-derived mesenchymal cells are detected by monoclonal antibodies. *Bone* 1992;**13**:69–80.
21 M. C. Galmiche, V. E. Koteliansky, J. Briere, *et al.* Stromal cells from human long-term marrow cultures are mesenchymal cells that differentiate following a vascular smooth muscle differentiation pathway. *Blood* 1993;**82**:66–76.
22 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.
23 K. Le Blanc, C. Tammik, K. Rosendahl, *et al.* HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;**31**:890–896.
24 V. Sordi, M. L. Malosio, F. Marchesi, *et al.* Bone marrow mesenchymal stem cells express a restricted set of functionally active chemokine receptors capable of promoting migration to pancreatic islets. *Blood* 2005;**106**:419–427.
25 X. Wang, R. Bie, Y. Sun, *et al.* The architecture of an automatic eHealth platform with mobile client for cerebrovascular disease detection. *JMIR Mhealth Uhealth* 2013;**1**:e20.

26 R. Gopurappilly, R. Pal, M. K. Mamidi, *et al.* Stem cells in stroke repair: current success and future prospects. *CNS Neurol Disord Drug Targets* 2011;**10**:741–756.

27 Y. Li, M. Chopp Cellular approaches to stroke recovery. In: Cramer S.C., Nudo R.J. (eds), *Brain Repair after Stroke*. Cambridge University Press: Cambridge, 2010; p. 267.

28 J. Wu, Z. Sun, H. S. Sun, *et al.* Intravenously administered bone marrow cells migrate to damaged brain tissue and improve neural function in ischemic rats. *Cell Transplant* 2008;**16**:993–1005.

29 J. Zhang, Y. Li, J. Chen, *et al.* Expression of insulin-like growth factor 1 and receptor in ischemic rats treated with human marrow stromal cells. *Brain Res* 2004;**1030**:19–27.

30 S. W. Yoo, S. S. Kim, S. Y. Lee, *et al.* Mesenchymal stem cells promote proliferation of endogenous neural stem cells and survival of newborn cells in a rat stroke model. *Exp Mol Med* 2008;**40**:387–397.

31 F. Guo, S. Lv, Y. Lou, *et al.* Bone marrow stromal cells enhance the angiogenesis in ischaemic cortex after stroke: involvement of notch signalling. *Cell Biol Int* 2012;**36**:997–1004.

32 A. Willing, M. Shahaduzzaman. Delivery routes for cell therapy in stroke. In: Jolkkonen J., Walczak P. (eds), *Cell-based Therapies in Stroke*. Springer: Vienna, 2013; pp. 15–28.

33 D. Kondziolka, G. K. Steinberg, L. Wechsler, *et al.* Neurotransplantation for patients with subcortical motor stroke: a phase 2 randomized trial. *J Neurosurg* 2005;**103**:38–45.

34 J. S. Lee, J. M. Hong, G. J. Moon, *et al.* A long-term follow-up study of intravenous autologous mesenchymal stem cell transplantation in patients with ischemic stroke. *Stem Cells* 2010;**28**:1099–1106.

35 V. Battistella, G. R. de Freitas, L. M. da Fonseca, *et al.* Safety of autologous bone marrow mononuclear cell transplantation in patients with nonacute ischemic stroke. *Regen Med* 2011;**6**:45–52.

36 F. Moniche, A. Gonzalez, J. R. Gonzalez-Marcos, *et al.* Intra-arterial bone marrow mononuclear cells in ischemic stroke: a pilot clinical trial. *Stroke* 2012;**43**:2242–2244.

37 L. Wang, Z. Lin, B. Shao, *et al.* Therapeutic applications of bone marrow-derived stem cells in ischemic stroke. *Neurol Res* 2013;**35**:470–478.

38 V. Misra, M. M. Ritchie, L. L. Stone, *et al.* Stem cell therapy in ischemic stroke: role of IV and intra-arterial therapy. *Neurology* 2012;**79**:S207–S212.

39 Y. Li, M. Chopp, J. Chen, *et al.* Intrastriatal transplantation of bone marrow nonhematopoietic cells improves functional recovery after stroke in adult mice. *J Cereb Blood Flow Metab* 2000;**20**:1311–1319.

40 H. Shichinohe, S. Kuroda, J. B. Lee, *et al. In vivo* tracking of bone marrow stromal cells transplanted into mice cerebral infarct by fluorescence optical imaging. *Brain Res Brain Res Protoc* 2004;**13**:166–175.

41 H. Shichinohe, T. Ishihara, K. Takahashi, *et al.* Bone marrow stromal cells rescue ischemic brain by trophic effects and phenotypic change toward neural cells. *Neurorehabil Neural Repair* 2015;**29**:80–89.

42 H. Irons, J. G. Lind, C. G. Wakade, *et al.* Intracerebral xenotransplantation of GFP mouse bone marrow stromal cells in intact and stroke rat brain: graft survival and immunologic response. *Cell Transplant* 2004;**13**:283–294.

43 J. Chen, Y. Li, L. Wang, *et al.* Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001;**32**:1005–1011.

44 M. Miyamoto, S. Kuroda, S. Zhao, *et al.* Bone marrow stromal cell transplantation enhances recovery of local glucose metabolism after cerebral infarction in rats: a serial $^{18}$F-FDG PET study. *J Nucl Med* 2013;**54**:145–150.

45 J. R. Chen, G. Y. Cheng, C. C. Sheu, *et al.* Transplanted bone marrow stromal cells migrate, differentiate and improve motor function in rats with experimentally induced cerebral stroke. *J Anat* 2008;**213**:249–258.

46 N. Liu, R. Chen, H. Du, *et al.* Expression of IL-10 and TNF-α in rats with cerebral infarction after transplantation with mesenchymal stem cells. *Cell Mol Immunol* 2009;**6**:207–213.

47 L. R. Zhao, W. M. Duan, M. Reyes, *et al.* Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 2002;**174**:11–20.

48 C. Zhang, Y. Li, J. Chen, Q. *et al.* Bone marrow stromal cells upregulate expression of bone morphogenetic proteins 2 and 4, gap junction protein connexin-43 and synaptophysin after stroke in rats. *Neuroscience* 2006;**141**:687–695.

49 Y. Li, J. Chen, L. Wang, *et al.* Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* 2001;**56**:1666–1672.

50 L. H. Shen, Y. Li, J. Chen, *et al.* Intracarotid transplantation of bone marrow stromal cells increases axon-myelin remodeling after stroke. *Neuroscience* 2006;**137**:393–399.

51 E. Keimpema, M. R. Fokkens, Z. Nagy, *et al.* Early transient presence of implanted bone marrow stem cells reduces lesion size after cerebral ischaemia in adult rats. *Neuropathol Appl Neurobiol* 2009;**35**:89–102.

52 L. Wang, Y. Li, J. Chen, *et al.* Ischemic cerebral tissue and MCP-1 enhance rat bone marrow stromal cell migration in interface culture. *Exp Hematol* 2002;**30**:831–836.

53 J. Chen, Z. G. Zhang, Y. Li, *et al.* Intravenous administration of human bone marrow stromal cells induces angiogenesis in the ischemic boundary zone after stroke in rats. *Circ Res* 2003;**92**: 692–629.

54 J. Chen, Y. Li, R. Zhang, *et al.* Combination therapy of stroke in rats with a nitric oxide donor and human bone marrow stromal cells enhances angiogenesis and neurogenesis. *Brain Res* 2004;**1005**: 21–28.

55 J. Chen, Y. Li, M. Katakowski, *et al.* Intravenous bone marrow stromal cell therapy reduces apoptosis and promotes endogenous cell proliferation after stroke in female rat. *J Neurosci Res* 2003;**73**:778–786.

56 H. Xin, Y. Li, Y. Cui, *et al.* Systemic administration of exosomes released from mesenchymal stromal cells promote functional recovery and neurovascular plasticity after stroke in rats. *J Cereb Blood Flow Metab* 2013;**33**:1711–1715.

57 T. Okazaki, T. Magaki, M. Takeda, *et al.* Intravenous administration of bone marrow stromal cells increases survivin and Bcl-2 protein expression and improves sensorimotor function following ischemia in rats. *Neurosci Lett* 2008;**430**:109–114.

58 W. Zheng, O. Honmou, K. Miyata, *et al.* Therapeutic benefits of human mesenchymal stem cells derived from bone marrow after global cerebral ischemia. *Brain Res* 2010;**1310**:8–16.

59 H. Shichinohe, T. Yamauchi, H. Saito, *et al.* Bone marrow stromal cell transplantation enhances recovery of motor function after lacunar stroke in rats. *Acta Neurobiol Exp (Wars)* 2013;**73**:354–363.

60 S. Ma, D. Zhong, H. Chen, *et al.* The immunomodulatory effect of bone marrow stromal cells (BMSCs) on interleukin (IL)-23/IL-17-mediated ischemic stroke in mice. *J Neuroimmunol* 2013;**257**: 28–35.

61 M. Song, O. Mohamad, X. Gu, *et al.* Restoration of intracortical and thalamocortical circuits after transplantation of bone marrow mesenchymal stem cells into the ischemic brain of mice. *Cell Transplant* 2013;**22**:2001–2015.

62 N. Liu, K. Deguchi, T. Yamashita, *et al.* Intracerebral transplantation of bone marrow stromal cells ameliorates tissue plasminogen activator-induced brain damage after cerebral ischemia in mice detected by *in vivo* and *ex vivo* optical imaging. *J Neurosci Res* 2012;**90**:2086–2093.

63 M. Ito, S. Kuroda, T. Sugiyama, *et al.* Transplanted bone marrow stromal cells protect neurovascular units and ameliorate brain damage in stroke-prone spontaneously hypertensive rats. *Neuropathology* 2012;**32**:522–533.

64 M. Kawabori, S. Kuroda, T. Sugiyama, *et al.* Intracerebral, but not intravenous, transplantation of bone marrow stromal cells enhances functional recovery in rat cerebral infarct: an optical imaging study. *Neuropathology* 2012;**32**:217–226.

65 T. Sugiyama, S. Kuroda, Y. Takeda, *et al.* Therapeutic impact of human bone marrow stromal cells expanded by animal serum-free medium for cerebral infarct in rats. *Neurosurgery* 2011;**68**: 1733–1742, discussion 1742.

66 L. H. Shen, Y. Li, M. Chopp. Astrocytic endogenous glial cell derived neurotrophic factor production is enhanced by bone marrow stromal cell transplantation in the ischemic boundary zone after stroke in adult rats. *Glia* 2010;**58**:1074–1081.

67 Z. Liu, Y. Li, Z. G. Zhang, *et al.* Bone marrow stromal cells enhance inter- and intracortical axonal connections after ischemic stroke in adult rats. *J Cereb Blood Flow Metab* 2010;**30**:1288–1295.

68 H. Xin, M. Chopp, L. H. Shen, *et al.* Multipotent mesenchymal stromal cells decrease transforming growth factor β1 expression in microglia/macrophages and down-regulate plasminogen activator inhibitor 1 expression in astrocytes after stroke. *Neurosci Lett* 2013;**542**:81–86.

69 B. Mitkari, F. Nitzsche, E. Kerkela, *et al.* Human bone marrow mesenchymal stem/stromal cells produce efficient localization in the brain and enhanced angiogenesis after intra-arterial delivery in rats with cerebral ischemia, but this is not translated to behavioral recovery. *Behav Brain Res* 2014;**259**:50–59.

70 S. W. Yoo, D. Y. Chang, H. S. Lee, *et al.* Immune following suppression mesenchymal stem cell transplantation in the ischemic brain is mediated by TGF-β. *Neurobiol Dis* 2013;**58**:249–257.

71 X. Ding, Y. Li, Z. Liu, *et al.* The sonic hedgehog pathway mediates brain plasticity and subsequent functional recovery after bone marrow stromal cell treatment of stroke in mice. *J Cereb Blood Flow Metab* 2013;**33**:1015–1024.

72 A. Moisan, N. Pannetier, E. Grillon, *et al.* Intracerebral injection of human mesenchymal stem cells impacts cerebral microvasculature after experimental stroke: MRI study. *NMR Biomed* 2012;**25**: 1340–1348.

73 Z. Liu, Y. Li, R. L. Zhang, *et al.* Bone marrow stromal cells promote skilled motor recovery and enhance contralesional axonal connections after ischemic stroke in adult mice. *Stroke* 2011;**42**:740–744.

74 K. Wakabayashi, A. Nagai, A. M. Sheikh, *et al.* Transplantation of human mesenchymal stem cells promotes functional improvement and increased expression of neurotrophic factors in a rat focal cerebral ischemia model. *J Neurosci Res* 2010;**88**:1017–1025.

75 M. Yang, X. Wei, J. Li, *et al.* Changes in host blood factors and brain glia accompanying the functional recovery after systemic administration of bone marrow stem cells in ischemic stroke rats. *Cell Transplant* 2010;**19**:1073–1084.

76 C. V. Borlongan, J. G. Lind, O. Dillon-Carter, *et al.* Bone marrow grafts restore cerebral blood flow and blood brain barrier in stroke rats. *Brain Res* 2004;**1010**:108–116.

77 D. R. Yavagal, B. Lin, A. P. Raval, *et al.* Efficacy and dose-dependent safety of intra-arterial delivery of mesenchymal stem cells in a rodent stroke model. *PLoS ONE* 2014;**9**:e93735.

78 D. K. Jang, S. I. Park, Y. M. Han, *et al.* Motor-evoked potential confirmation of functional improvement by transplanted bone marrow mesenchymal stem cell in the ischemic rat brain. *J Biomed Biotechnol* 2011;**2011**:238409.

79 Z. Yang, L. Zhu, F. Li, *et al.* Bone marrow stromal cells as a therapeutic treatment for ischemic stroke. *Neurosci Bull* 2014;**30**:524–534.

80 K. Jin, Y. Sun, L. Xie, *et al.* Comparison of ischemia-directed migration of neural precursor cells after intrastriatal, intraventricular, or intravenous transplantation in the rat. *Neurobiol Dis* 2005;**18**:366–374.

81 P. Walczak, J. Zhang, A. A. Gilad, *et al.* Dual-modality monitoring of targeted intraarterial delivery of mesenchymal stem cells after transient ischemia. *Stroke* 2008;**39**:1569–1574.

82 H. Wan, F. Li, L. Zhu, *et al.* Update on therapeutic mechanism for bone marrow stromal cells in ischemic stroke. *J Mol Neurosci* 2014;**52**:177–185.

83 M. Kawabori, S. Kuroda, M. Ito, *et al.* Timing and cell dose determine therapeutic effects of bone marrow stromal cell transplantation in rat model of cerebral infarct. *Neuropathology* 2013;**33**:140–148.

84 R. Guzman, R. Choi, A. Gera, *et al.* Intravascular cell replacement therapy for stroke. *Neurosurg Focus* 2008;**24**:E15.

85 T. M. Bliss, R. H. Andres, G. K. Steinberg. Optimizing the success of cell transplantation therapy for stroke. *Neurobiol Dis* 2010;**37**:275–283.

86 C. Wang, F. Li, Y. Guan, *et al.* Bone marrow stromal cells combined with oxiracetam influences the expression of B-cell lymphoma 2 in rats with ischemic stroke. *J Stroke Cerebrovasc Dis* 2014;**23**: 2591–2597.

87 J. Suzuki, M. Sasaki, K. Harada, *et al.* Bilateral cortical hyperactivity detected by fMRI associates with improved motor function following intravenous infusion of mesenchymal stem cells in a rat stroke model. *Brain Res* 2013;**1497**:15–22.

88 U. M. Fischer, M. T. Harting, F. Jimenez, *et al.* Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009;**18**:683–692.

89 A. Hicks, J. Jolkkonen. Challenges and possibilities of intravascular cell therapy in stroke. *Acta Neurobiol Exp (Wars)* 2009;**69**: 1–11.

90 G. V. Goldmacher, R. Nasser, D. Y. Lee, *et al.* Tracking transplanted bone marrow stem cells and their effects in the rat MCAO stroke model. *PLoS ONE* 2013;**8**:e60049.

91 M. Modo, R. P. Stroemer, E. Tang, *et al.* Effects of implantation site of stem cell grafts on behavioral recovery from stroke damage. *Stroke* 2002;**33**:2270–2278.

92 L. Guo, J. Ge, S. Wang, *et al.* A novel method for efficient delivery of stem cells to the ischemic brain. *Stem Cell Res Ther* 2013;**4**:116.

93 S. Ishizaka, N. Horie, K. Satoh, *et al.* Intra-arterial cell transplantation provides timing-dependent cell distribution and functional recovery after stroke. *Stroke* 2013;**44**:720–726.

94 B. Mitkari, E. Kerkela, J. Nystedt, *et al.* Intra-arterial infusion of human bone marrow-derived mesenchymal stem cells results in transient localization in the brain after cerebral ischemia in rats. *Exp Neurol* 2013;**239**:158–1562.

95 M. Janowski, A. Lyczek, C. Engels, *et al.* Cell size and velocity of injection are major determinants of the safety of intracarotid stem cell transplantation. *J Cereb Blood Flow Metab* 2013;**33**:921–927.

96 M. Ito, H. Shichinohe, K. Houkin, S. Kuroda. Application of cell sheet technology to bone marrow stromal cell transplantation for rat brain infarct. *J Tissue Eng Regen Med* 2014; doi: 10.1002/term.1920.

97 Y. Wang, J. Huang, Y. Li, G. Y. Yang. Roles of chemokine CXCL12 and its receptors in ischemic stroke. *Curr Drug Targets* 2012;**13**:166–172.

98 X. Yu, D. Chen, Y. Zhang, *et al.* Overexpression of CXCR4 in mesenchymal stem cells promotes migration, neuroprotection and angiogenesis in a rat model of stroke. *J Neurol Sci* 2012;**316**:141–149.

99 Y. Wang, W. Fu, S. Zhang, *et al.* CXCR7 receptor promotes SDF-1α-induced migration of bone marrow mesenchymal stem cells in the transient cerebral ischemia/reperfusion rat hippocampus. *Brain Res* 2014;**1575**:78–86.

100 Y. Wang, Y. Deng, G. Q. Zhou. SDF-1α/CXCR4-mediated migration of systemically transplanted bone marrow stromal cells towards ischemic brain lesion in a rat model. *Brain Res* 2008;**1195**:104–112.

101 I. Petit, M. Szyper-Kravitz, A. Nagler, *et al.* G-CSF induces stem cell mobilization by decreasing bone marrow SDF-1 and up-regulating CXCR4. *Nat Immunol* 2002;**3**:687–694.

102 W. C. Shyu, S. Z. Lin, P. S. Yen, *et al.* Stromal cell-derived factor-1α promotes neuroprotection, angiogenesis, and mobilization/homing of bone marrow-derived cells in stroke rats. *J Pharmacol Exp Ther* 2008;**324**:834–849.

103 G. Yilmaz, S. Vital, C. E. Yilmaz, *et al.* Selectin-mediated recruitment of bone marrow stromal cells in the postischemic cerebral microvasculature. *Stroke* 2011;**42**:806–811.

104 Y. S. Lee, C. C. Chio, C. P. Chang, *et al.* Long course hyperbaric oxygen stimulates neurogenesis and attenuates inflammation after ischemic stroke. *Mediators Inflamm* 2013;**2013**:512978.

105 Z. Gong, H. Ran, S. Wu, *et al.* Ultrasound-microbubble transplantation of bone marrow stromal cells improves neurological function after forebrain ischemia in adult mice. *Cell Biochem Biophys* 2014;**70**:499–504.

106 K. Deguchi, K. Tsuru, T. Hayashi, *et al.* Implantation of a new porous gelatin–siloxane hybrid into a brain lesion as a potential scaffold for tissue regeneration. *J Cereb Blood Flow Metab* 2006;**26**:1263–1273.

107 H. Zhang, T. Kamiya, T. Hayashi, *et al.* Gelatin–siloxane hybrid scaffolds with vascular endothelial growth factor induces brain tissue regeneration. *Curr Neurovasc Res* 2008;**5**:112–117.

108 H. Yasuda, S. Kuroda, H. Shichinohe, *et al.* Effect of biodegradable fibrin scaffold on survival, migration, and differentiation of transplanted bone marrow stromal cells after cortical injury in rats. *J Neurosurg* 2010;**112**:336–344.

109 T. Osanai, S. Kuroda, H. Yasuda, *et al.* Noninvasive transplantation of bone marrow stromal cells for ischemic stroke: preliminary study with a thermoreversible gelation polymer hydrogel. *Neurosurgery* 2010;**66**:1140–1147, discussion 1147.

110 K. Abe, T. Yamashita, S. Takizawa, *et al.* Stem cell therapy for cerebral ischemia: from basic science to clinical applications. *J Cereb Blood Flow Metab* 2012;**32**:1317–1331.

111 M. Hoehn, E. Kustermann, J. Blunk, *et al.* Monitoring of implanted stem cell migration *in vivo*: a highly resolved *in vivo* magnetic resonance imaging investigation of experimental stroke in rat. *Proc Natl Acad Sci U S A* 2002;**99**:16267–16272.

112 M. Ito, S. Kuroda, T. Sugiyama, *et al.* Validity of bone marrow stromal cell expansion by animal serum-free medium for cell transplantation therapy of cerebral infarct in rats – a serial MRI study. *Transl Stroke Res* 2011;**2**:294–306.

113 S. S. Lu, S. Liu, Q. Q. Zu, *et al. In vivo* MR imaging of intraarterially delivered magnetically labeled mesenchymal stem cells in a canine stroke model. *PLoS ONE* 2013;**8**:e54963.

114 J. T. Rosenberg, K. L. Sellgren, A. Sachi-Kocher, *et al.* Magnetic resonance contrast and biological effects of intracellular superparamagnetic iron oxides on human mesenchymal stem cells with long-term culture and hypoxic exposure. *Cytotherapy* 2013;**15**:307–322.

115 O. Detante, S. Valable, F. de Fraipont, *et al.* Magnetic resonance imaging and fluorescence labeling of clinical-grade mesenchymal stem cells without impacting their phenotype: study in a rat model of stroke. *Stem Cells Transl Med* 2012;**1**:333–341.

116 E. Tarulli, J. D. Chaudhuri, V. Gretka, *et al.* Effectiveness of micron-sized superparamagnetic iron oxide particles as markers for detection of migration of bone marrow-derived mesenchymal stromal cells in a stroke model. *J Magn Reson Imaging* 2013;**37**:1409–1418.

117 P. L. Correa, C. T. Mesquita, R. M. Felix, *et al.* Assessment of intra-arterial injected autologous bone marrow mononuclear cell distribution by radioactive labeling in acute ischemic stroke. *Clin Nucl Med* 2007;**32**:839–841.

118 K. Mori, J. Iwata, M. Miyazaki, *et al.* Functional recovery of neuronal activity in rat whisker-barrel cortex sensory pathway from freezing injury after transplantation of adult bone marrow stromal cells. *J Cereb Blood Flow Metab* 2005;**25**:887–898.

119 T. Wu, J. Lang, X. Sun, *et al.* Monitoring bone marrow stem cells with a reporter gene system in experimental middle cerebral artery occlusion rat models. *J Nucl Med* 2013;**54**:984–989.

120 H. Saito, K. Magota, S. Zhao, *et al.* $^{123}$I-iomazenil single photon emission computed tomography visualizes recovery of neuronal integrity by bone marrow stromal cell therapy in rat infarct brain. *Stroke* 2013;**44**:2869–2874.

121 T. Sugiyama, S. Kuroda, T. Osanai, *et al.* Near-infrared fluorescence labeling allows noninvasive tracking of bone marrow stromal cells transplanted into rat infarct brain. *Neurosurgery* 2011;**68**:1036–1047, discussion 1047.

122 T. Osanai, S. Kuroda, T. Sugiyama, *et al.* Therapeutic effects of intra-arterial delivery of bone marrow stromal cells in traumatic brain injury of rats – *in vivo* cell tracking study by near-infrared fluorescence imaging. *Neurosurgery* 2012;**70**:435–444, discussion 444.

123 K. Li, M. Yamamoto, S. J. Chan, *et al.* Organic nanoparticles with aggregation-induced emission for tracking bone marrow stromal cells in the rat ischemic stroke model. *Chem Commun (Camb)* 2014;**50**:15136–15139.

124 Y. Li, M. Chopp. Marrow stromal cell transplantation in stroke and traumatic brain injury. *Neurosci Lett* 2009;**456**:120–123.

125 Y. Zhao, Y. Guan, Y. Xu, *et al.* Sodium ferulate combined with bone marrow stromal cell treatment ameliorating rat brain ischemic injury after stroke. *Brain Res* 2012;**1450**:157–165.

126 X. Cui, J. Chen, A. Zacharek, *et al.* Nitric oxide donor upregulation of stromal cell-derived factor-1/chemokine (CXC motif) receptor 4 enhances bone marrow stromal cell migration into ischemic brain after stroke. *Stem Cells* 2007;**25**:2777–2785.

127 Y. Li, J. Chen, X. G. Chen, *et al.* Human marrow stromal cell therapy for stroke in rat: neurotrophins and functional recovery. *Neurology* 2002;**59**:514–523.

128 R. Qu, Y. Li, Q. Gao, *et al.* Neurotrophic and growth factor gene expression profiling of mouse bone marrow stromal cells induced by ischemic brain extracts. *Neuropathology* 2007;**27**:355–363.

129 M. Hokari, S. Kuroda, Y. Chiba, *et al.* Synergistic effects of granulocyte-colony stimulating factor on bone marrow stromal cell transplantation for mice cerebral infarct. *Cytokine* 2009;**46**:260–266.

130 N. Li, P. Wang, X. L. Ma, *et al.* Effect of bone marrow stromal cell transplantation on neurologic function and expression of VEGF in rats with focal cerebral ischemia. *Mol Med Rep* 2014;**10**:2299–2305.

131 Y. Jia, J. Zhou, Y. Tai, Y. Wang. TRPC channels promote cerebellar granule neuron survival. *Nat Neurosci* 2007;**10**:559–567.

132 W. Du, J. Huang, H. Yao, *et al.* Inhibition of TRPC6 degradation suppresses ischemic brain damage in rats. *J Clin Invest* 2010;**120**:3480–3492.

133 G. Yilmaz, J. S. Alexander, C. Erkuran Yilmaz, D. N. Granger. Induction of neuro-protective/regenerative genes in stem cells infiltrating post-ischemic brain tissue. *Exp Transl Stroke Med* 2010;**2**:11.

134 Y. Sun, K. Jin, L. Xie, *et al.* VEGF-induced neuroprotection, neurogenesis, and angiogenesis after focal cerebral ischemia. *J Clin Invest* 2003;**111**:1843–1851.

135 N. Horie, M. P. Pereira, K. Niizuma, *et al.* Transplanted stem cell-secreted vascular endothelial growth factor effects poststroke recovery, inflammation, and vascular repair. *Stem Cells* 2011;**29**:274–285.

136 Y. Ma, A. Zechariah, Y. Qu, D. M. Hermann. Effects of vascular endothelial growth factor in ischemic stroke. *J Neurosci Res* 2012;**90**:1873–1882.

137 H. Xin, Y. Li, X. Chen, M. Chopp. Bone marrow stromal cells induce BMP2/4 production in oxygen–glucose-deprived astrocytes, which promotes an astrocytic phenotype in adult subventricular progenitor cells. *J Neurosci Res* 2006;**83**:1485–1493.

138 A. Zacharek, A. Shehadah, J. Chen, *et al.* Comparison of bone marrow stromal cells derived from stroke and normal rats for stroke treatment. *Stroke* 2010;**41**:524–530.

139 Y. S. Kim, M. Y. Noh, K. A. Cho, *et al.* Hypoxia/reoxygenation-preconditioned human bone marrow-derived mesenchymal stromal cells rescue ischemic rat cortical neurons by enhancing trophic factor release. *Mol Neurobiol* 2015;**52**:792–803.

140 A. Bronckaers, P. Hilkens, W. Martens, *et al.* Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis. *Pharmacol Ther* 2014;**143**:181–196.

141 Y. Omori, O. Honmou, K. Harada, *et al.* Optimization of a therapeutic protocol for intravenous injection of human mesenchymal stem cells after cerebral ischemia in adult rats. *Brain Res* 2008:**1236**:30–38.

142 L. H. Shen, Y. Li, J. Chen, *et al.* Therapeutic benefit of bone marrow stromal cells administered 1 month after stroke. *J Cereb Blood Flow Metab* 2007;**27**:6–13.

143 K. Komatsu, O. Honmou, J. Suzuki, *et al.* Therapeutic time window of mesenchymal stem cells derived from bone marrow after cerebral ischemia. *Brain Res* 2010;**1334**:84–92.

144 X. Cui, J. Chen, A. Zacharek, *et al.* Treatment of stroke with (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate and bone marrow stromal cells upregulates angiopoietin-1/Tie2 and enhances neovascularization. *Neuroscience* 2008;**156**:155–164.

145 L. Wei, J. L. Fraser, Z. Y. Lu, *et al.* Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats. *Neurobiol Dis* 2012;**46**:635–645.

146 T. Onda, O. Honmou, K. Harada, *et al.* Therapeutic benefits by human mesenchymal stem cells (hMSCs) and Ang-1 gene-modified hMSCs after cerebral ischemia. *J Cereb Blood Flow Metab* 2008;**28**:329–340.

147 W. C. Shyu, C. P. Chen, S. Z. Lin, *et al.* Efficient tracking of non-iron-labeled mesenchymal stem cells with serial MRI in chronic stroke rats. *Stroke* 2007;**38**:367–374.

148 E. Pacary, H. Legros, S. Valable, *et al.* Synergistic effects of $CoCl_2$ and ROCK inhibition on mesenchymal stem cell differentiation into neuron-like cells. *J Cell Sci* 2006;**119**:2667–2678.

149 T. Yasuhara, N. Matsukawa, K. Hara, *et al.* Notch-induced rat and human bone marrow stromal cell grafts reduce ischemic cell loss and ameliorate behavioral deficits in chronic stroke animals. *Stem Cells Dev* 2009;**18**:1501–1514.

150 T. Mimura, M. Dezawa, H. Kanno, I. Yamamoto. Behavioral and histological evaluation of a focal cerebral infarction rat model transplanted with neurons induced from bone marrow stromal cells. *J Neuropathol Exp Neurol* 2005;**64**:1108–1117.

151 J. S. Heo, S. M. Choi, H. O. Kim, *et al.* Neural transdifferentiation of human bone marrow mesenchymal stem cells on hydrophobic polymer-modified surface and therapeutic effects in an animal model of ischemic stroke. *Neuroscience* 2013;**238**:305–318.

152 J. Tu, F. Yang, J. Wan, *et al.* Light-controlled astrocytes promote human mesenchymal stem cells toward neuronal differentiation and improve the neurological deficit in stroke rats. *Glia* 2014;**62**:106–121.

153 Y. Liu, Y. Zhang, L. Lin, *et al.* Effects of bone marrow-derived mesenchymal stem cells on the axonal outgrowth through activation of PI3K/AKT signaling in primary cortical neurons followed oxygen–glucose deprivation injury. *PLoS ONE* 2013;**8**:e78514.

154 Y. Li, K. McIntosh, J. Chen, *et al.* Allogeneic bone marrow stromal cells promote glial-axonal remodeling without immunologic sensitization after stroke in rats. *Exp Neurol* 2006;**198**:313–325.

155 H. Xin, Y. Li, B. Buller, *et al.* Exosome-mediated transfer of miR-133b from multipotent mesenchymal stromal cells to neural cells contributes to neurite outgrowth. *Stem Cells* 2012;**30**:1556–1564.

156 H. Xin, Y. Li, Z. Liu, *et al.* MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent

mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem Cells* 2013;**31**:2737–2746.

157 Y. B. Deng, W. B. Ye, Z. Z. Hu, *et al.* Intravenously administered BMSCs reduce neuronal apoptosis and promote neuronal proliferation through the release of VEGF after stroke in rats. *Neurol Res* 2010;**32**:148–156.

158 G. Tang, Y. Liu, Z. Zhang, *et al.* Mesenchymal stem cells maintain blood–brain barrier integrity by inhibiting aquaporin-4 up-regulation after cerebral ischemia. *Stem Cells* 2014;**32**:3150–3162.

159 M. L. Calió, D. S. Marinho, G. M. Ko, *et al.* Transplantation of bone marrow mesenchymal stem cells decreases oxidative stress, apoptosis, and hippocampal damage in brain of a spontaneous stroke model. *Free Radic Biol Med* 2014;**70**:141–154.

160 P. Huang, N. Gebhart, E. Richelson, *et al.* Mechanism of mesenchymal stem cell-induced neuron recovery and anti-inflammation. *Cytotherapy* 2014;**16**:1336–1344.

161 F. Scheibe, O. Klein, J. Klose, J. Priller. Mesenchymal stromal cells rescue cortical neurons from apoptotic cell death in an *in vitro* model of cerebral ischemia. *Cell Mol Neurobiol* 2012;**32**:567–576.

162 K. Kurozumi, K. Nakamura, T. Tamiya, *et al.* BDNF gene-modified mesenchymal stem cells promote functional recovery and reduce infarct size in the rat middle cerebral artery occlusion model. *Mol Ther* 2004;**9**:189–197.

163 M. Z. Zhao, N. Nonoguchi, N. Ikeda, *et al.* Novel therapeutic strategy for stroke in rats by bone marrow stromal cells and *ex vivo* HGF gene transfer with HSV-1 vector. *J Cereb Blood Flow Metab* 2006;**26**:1176–1188.

164 B. Engelhardt, L. Sorokin. The blood–brain and the blood–cerebrospinal fluid barriers: function and dysfunction. *Semin Immunopathol* 2009;**31**:497–511.

165 C. Kleinschnitz, N. Schwab, P. Kraft, *et al.* Early detrimental T-cell effects in experimental cerebral ischemia are neither related to adaptive immunity nor thrombus formation. *Blood* 2010;**115**:3835–3842.

166 M. Krampera, S. Glennie, J. Dyson, *et al.* Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;**101**:3722–3729.

167 L. Q. Wang, Z. Z. Lin, H. X. Zhang, *et al.* Timing and dose regimens of marrow mesenchymal stem cell transplantation affect the outcomes and neuroinflammatory response after ischemic stroke. *CNS Neurosci Ther* 2014;**20**:317–326.

168 H. Ooboshi, S. Ibayashi, T. Shichita, *et al.* Postischemic gene transfer of interleukin-10 protects against both focal and global brain ischemia. *Circulation* 2005;**111**:913–919.

169 F. Scheibe, J. Ladhoff, J. Huck, *et al.* Immune effects of mesenchymal stromal cells in experimental stroke. *J Cereb Blood Flow Metab* 2012;**32**:1578–1588.

170 N. Ikeda, N. Nonoguchi, M. Z. Zhao, *et al.* Bone marrow stromal cells that enhanced fibroblast growth factor-2 secretion by herpes simplex virus vector improve neurological outcome after transient focal cerebral ischemia in rats. *Stroke* 2005;**36**:2725–2730.

171 T. Nomura, O. Honmou, K. Harada, *et al.* IV infusion of brain-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in adult rat. *Neuroscience* 2005;**136**:161–169.

172 Y. Horita, O. Honmou, K. Harada, *et al.* Intravenous administration of glial cell line-derived neurotrophic factor gene-modified human mesenchymal stem cells protects against injury in a cerebral ischemia model in the adult rat. *J Neurosci Res* 2006;**84**:1495–1504.

173 H. Liu, O. Honmou, K. Harada, *et al.* Neuroprotection by PLGF gene-modified human mesenchymal stem cells after cerebral ischaemia. *Brain* 2006;**129**:2734–2745.

174 L. Wei, C. M. Rovainen, T. A. Woolsey. Ministrokes in rat barrel cortex. *Stroke* 1995;**26**:1459–1462.

175 N. Wei, S. P. Yu, X. Gu, *et al.* Delayed intranasal delivery of hypoxic-preconditioned bone marrow mesenchymal stem cells enhanced cell homing and therapeutic benefits after ischemic stroke in mice. *Cell Transplant* 2013;**22**:977–991.

176 C. Chen, Y. Cheng, J. Chen. Transfection of Noggin in bone marrow stromal cells (BMSCs) enhances BMSC-induced functional outcome after stroke in rats. *J Neurosci Res* 2011;**89**:1194–1202.

177 J. Ding, Y. Cheng, S. Gao, J. Chen. Effects of nerve growth factor and Noggin-modified bone marrow stromal cells on stroke in rats. *J Neurosci Res* 2011;**89**:222–230.

178 T. Yamauchi, H. Saito, M. Ito, *et al.* Platelet lysate and granulocyte-colony stimulating factor serve safe and accelerated expansion of human bone marrow stromal cells for stroke therapy. *Transl Stroke Res* 2014;**5**:701–710.

179 W. Huang, X. Mo, C. Qin, *et al.* Transplantation of differentiated bone marrow stromal cells promotes motor functional recovery in rats with stroke. *Neurol Res* 2013;**35**:320–328.

180 T. Lai, M. Li, L. Zheng, *et al.* Over-expression of VEGF in marrow stromal cells promotes angiogenesis in rats with cerebral infarction via the synergistic effects of VEGF and Ang-2. *J Huazhong Univ Sci Technol Med Sci* 2012;**32**:724–731.

181 L. H. Shen, M. Ye, X. S. Ding, *et al.* Protective effects of MCI-186 on transplantation of bone marrow stromal cells in rat ischemic stroke model. *Neuroscience* 2012;**223**:315–324.

182 L. K. Tsai, Z. Wang, J. Munasinghe, *et al.* Mesenchymal stem cells primed with valproate and lithium robustly migrate to infarcted regions and facilitate recovery in a stroke model. *Stroke* 2011;**42**:2932–2939.

183 N. Liu, Y. Zhang, L. Fan, *et al.* Effects of transplantation with bone marrow-derived mesenchymal stem cells modified by Survivin on experimental stroke in rats. *J Transl Med* 2011;**9**:105.

184 X. Bao, M. Feng, J. Wei, *et al.* Transplantation of Flk-1$^{+}$ human bone marrow-derived mesenchymal stem cells promotes angiogenesis and neurogenesis after cerebral ischemia in rats. *Eur J Neurosci* 2011;**34**:87–98.

185 G. W. Cho, S. H. Koh, M. H. Kim, *et al.* The neuroprotective effect of erythropoietin-transduced human mesenchymal stromal cells in an animal model of ischemic stroke. *Brain Res* 2010;**1353**:1–13.

186 M. Hayase, M. Kitada, S. Wakao, *et al.* Committed neural progenitor cells derived from genetically modified bone marrow stromal cells ameliorate deficits in a rat model of stroke. *J Cereb Blood Flow Metab* 2009;**29**:1409–1420.

187 K. Toyama, O. Honmou, K. Harada, *et al.* Therapeutic benefits of angiogenetic gene-modified human mesenchymal stem cells after cerebral ischemia. *Exp Neurol* 2009;**216**:47–55.

188 C. Yang, H. Liu, D. Liu. Mutant hypoxia-inducible factor 1α modified bone marrow mesenchymal stem cells ameliorate cerebral ischemia. *Int J Mol Med* 2014;**34**:1622–1628.

189 S. J. Kim, G. J. Moon, W. H. Chang, *et al.* Intravenous transplantation of mesenchymal stem cells preconditioned with early phase

stroke serum: current evidence and study protocol for a randomized trial. *Trials* 2013;**14**:317.

190 M. J. Tsai, S. K. Tsai, B. R. Hu, *et al.* Recovery of neurological function of ischemic stroke by application of conditioned medium of bone marrow mesenchymal stem cells derived from normal and cerebral ischemia rats. *J Biomed Sci* 2014;**21**:5.

191 C. Krause, A. Guzman, P. Knaus. Noggin. *Int J Biochem Cell Biol* 2011;**43**:478–481.

192 H. Tang, Y. Xiang, X. Jiang, *et al.* Dual expression of hTERT and VEGF prolongs life span and enhances angiogenic ability of aged BMSCs. *Biochem Biophys Res Commun* 2013;**440**:502–508.

193 J. Chen, Y. Li, L. Wang, *et al.* Caspase inhibition by Z-VAD increases the survival of grafted bone marrow cells and improves functional outcome after MCAo in rats. *J Neurol Sci* 2002;**199**: 17–24.

194 Y. Zhao, W. Lai, Y. Xu, *et al.* Exogenous and endogenous therapeutic effects of combination sodium ferulate and bone marrow stromal cells (BMSCs) treatment enhance neurogenesis after rat focal cerebral ischemia. *Metab Brain Dis* 2013;**28**:655–666.

195 F. Tian, T. Yamashita, K. Deguchi, *et al. In vivo* optical imaging correlates with improvement of cerebral ischemia treated by intravenous bone marrow stromal cells (BMSCs) and edaravone. *Neurol Res* 2013;**35**:1051–108.

196 M. Kometani, M. Okada, E. Takemori, *et al.* Effect of oxiracetam on cerebrovascular impairment in rats. *Arzneimittelforschung* 1991;**41**:684–689.

197 S. Bilen, F. Pinarli, F. Ak, *et al.* Treatment efficacy with bone marrow derived mesenchymal stem cells and minocycline in rats after cerebral ischemic injury. *Stem Cell Rev* 2013;**9**:219–225.

198 G. Pirzad Jahromi, S. Seidi, S. S. Sadr, *et al.* Therapeutic effects of a combinatorial treatment of simvastatin and bone marrow stromal cells on experimental embolic stroke. *Basic Clin Pharmacol Toxicol* 2012;**110**:487–493.

199 Y. H. Zhao, Y. Guan, W. K. Wu. Potential advantages of a combination of Chinese medicine and bone marrow mesenchymal stem cell transplantation for removing blood stasis and stimulating neogenesis during ischemic stroke treatment. *J Tradit Chin Med* 2012;**32**:289–292.

200 Q. Zhang, Y. H. Zhao. Therapeutic angiogenesis after ischemic stroke: Chinese medicines, bone marrow stromal cells (BMSCs) and their combinational treatment. *Am J Chin Med* 2014;**42**:61–77.

201 A. T. Balseanu, A. M. Buga, B. Catalin, *et al.* Multimodal approaches for regenerative stroke therapies: combination of granulocyte colony-stimulating factor with bone marrow mesenchymal stem cells is not superior to G-CSF alone. *Front Aging Neurosci* 2014;**6**:130.

202 O. Y. Bang, J. S. Lee, P. H. Lee, G. Lee. Autologous mesenchymal stem cell transplantation in stroke patients. *Ann Neurol* 2005;**57**:874–882.

203 M. A. Friedrich, M. P. Martins, M. D. Araujo, *et al.* Intra-arterial infusion of autologous bone marrow mononuclear cells in patients with moderate to severe middle cerebral artery acute ischemic stroke. *Cell Transplant* 2012;**21**(Suppl 1):S13–S21.

204 O. Honmou, K. Houkin, T. Matsunaga, *et al.* Intravenous administration of auto serum-expanded autologous mesenchymal stem cells in stroke. *Brain* 2011;**134**:1790–1807.

205 S. I. Savitz, M. Fisher. Future of neuroprotection for acute stroke: in the aftermath of the SAINT trials. *Ann Neurol* 2007;**61**:396–402.

206 Y. Chiba, S. Kuroda, T. Osanai, *et al.* Impact of ageing on biological features of bone marrow stromal cells (BMSC) in cell transplantation therapy for CNS disorders: functional enhancement by granulocyte-colony stimulating factor (G-CSF). *Neuropathology* 2012;**32**:139–148.

207 P. J. Hanley, Z. Mei, A. G. Durett, *et al.* Efficient manufacturing of therapeutic mesenchymal stromal cells with the use of the Quantum cell expansion system. *Cytotherapy* 2014;**16**: 1048–1058.

208 J. Chen, X. Ye, T. Yan, *et al.* Adverse effects of bone marrow stromal cell treatment of stroke in diabetic rats. *Stroke* 2011;**42**:3551–3558.

209 X. Ye, T. Yan, M. Chopp, *et al.* Combination BMSC and Niaspan treatment of stroke enhances white matter remodeling and synaptic protein expression in diabetic rats. *Int J Mol Sci* 2013;**14**: 22221–22232.

210 T. Yan, X. Ye, M. Chopp, *et al.* Niaspan attenuates the adverse effects of bone marrow stromal cell treatment of stroke in type one diabetic rats. *PLoS ONE* 2013;**8**:e81199.

211 B. Steiner, M. Roch, N. Holtkamp, A. Kurtz. Systemically administered human bone marrow-derived mesenchymal stem home into peripheral organs but do not induce neuroprotective effects in the MCAo-mouse model for cerebral ischemia. *Neurosci Lett* 2012;**513**:25–30.

212 J. Boltze, B. Lukomska, J. Jolkkonen. Mesenchymal stromal cells in stroke: improvement of motor recovery or functional compensation? *J Cereb Blood Flow Metab* 2014;**34**:1420–1421.

213 L. Y. Qiao, F. J. Huang, M. Zhao, *et al.* A two-year follow-up study of co-transplantation with neural stem/precursor cells and mesenchymal stromal cells in ischemic stroke patients. *Cell Transplant* 2014;**23**(Suppl 1):S65–S72.

214 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**:e47559.

CHAPTER 49

# The role of mesenchymal stromal cells in spinal cord injury

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## 49.1 The central nervous system

The central nervous system (CNS) comprises the brain and spinal cord and is divided into white and grey matter. The white matter consists of axons (nerve fibers) and oligodendrocytes, while the grey matter consists chiefly of neurons (nerve cells). Both tissues include a number of glial cells, which are often referred to as the supporting cells of the CNS. Different forms of glial cells have different functions, some acting almost as scaffolding for neuroblasts to climb during neurogenesis, while others such as microglia are a specialized form of macrophage involved in the immune system of the brain as well as in the clearance of various metabolites from brain tissue. Oligodendrocytes are a type of neuroglia. Their main functions are to provides support and insulation to axons in the CNS. Astrocytes may be involved with both clearance of metabolites and transport of nutrients to neurons from the capillaries of the brain. When the CNS is injured, astrocytes will proliferate, causing gliosis, a form of neuronal scar tissue, lacking in functional neurons.

## 49.2 Spinal cord injury

Spinal cord injury (SCI) is one of the most devastating diseases that affects patients and their families for the rest of their lives. Most of the cases are caused by traffic accidents, falls, sports, and assaults and therefore often involve young people. The costs of SCI to society include a reduction in quality of life and productivity, lower life expectancy, and high medical costs. In 2007 the medical and related expenses for a 25-year-old tetraplegic patient in the USA were estimated at $3 million [1]. Patients with SCI also face increased risk of cardiovascular complications, deep-vein thrombosis, osteoporosis, pressure ulcers, autonomic dysreflexia, and neuropathic pain [2].

SCI is a complex event with a variety of accompanying factors. Mechanical trauma to the spinal cord triggers an immediate cascade of cellular and biochemical events that leads to lesion progression. Primary injury results in blood vessel disruption and extensive cell death, followed by edema, ischemia, and release of cytotoxic chemicals and proinflammatory cytokines (Figure 49.1). In response, macrophages, neutrophils, and other leukocytes are attracted to the lesion and are recruited to phagocytose cell debris and create a local inflammatory environment. Further lesions develop due to secondary processes, which include ischemia, lipid peroxidation, the production of free radicals, disruption of ion channels, axonal demyelination, necrosis, and apoptosis [3]. In a matter of days to weeks a fluid-filled cyst forms at the injury site surrounded by a glial scar, which consists mainly of reactive astrocytes. These astrocytes increase their metabolism and form processes, preventing the further spread of the aggressive environment both up and down the spinal cord [4,5]. They also secrete several inhibitory proteins, including chondroitin sulfate proteoglycans and axonal growth inhibitors (Figure 49.1). This barrier blocks axonal

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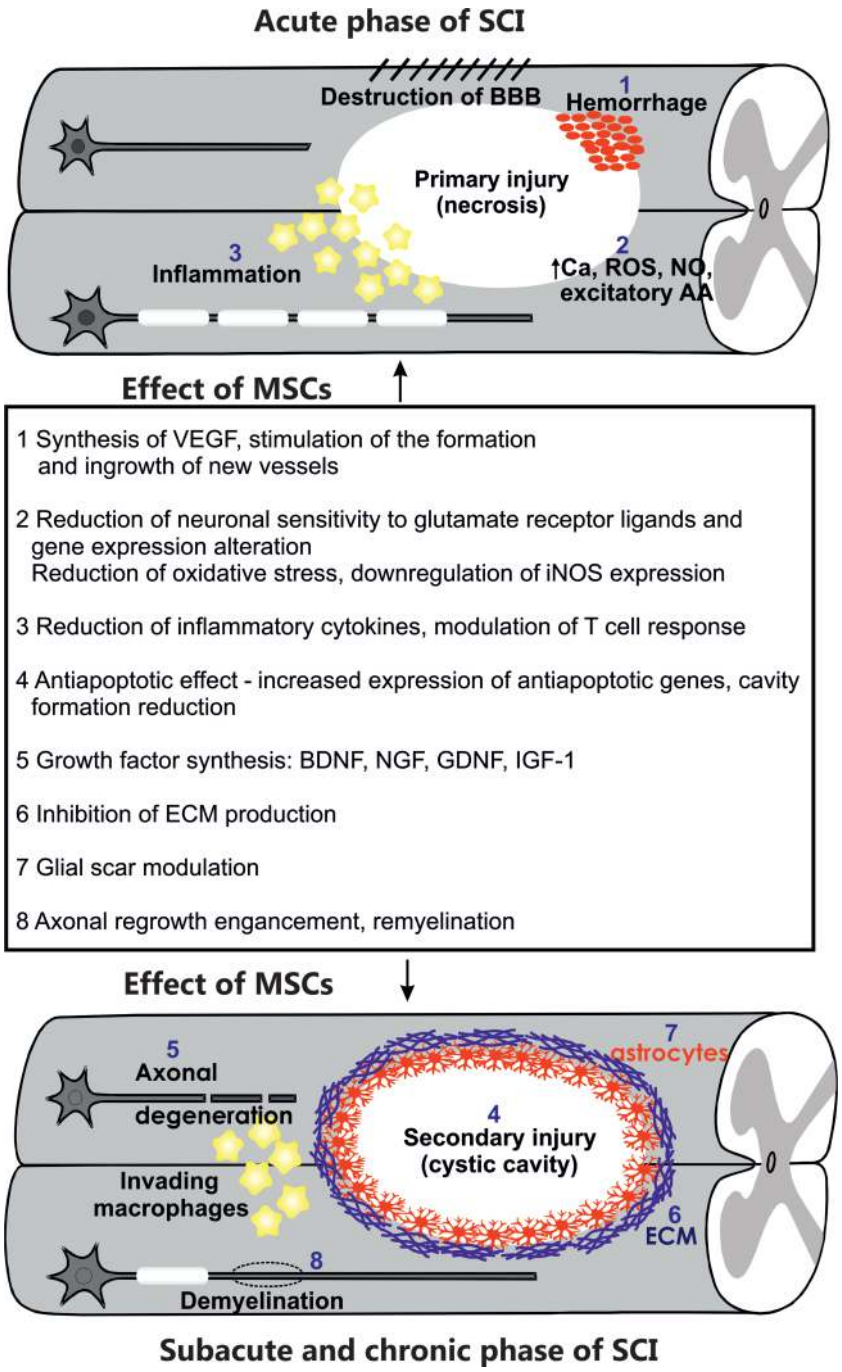



**Figure 49.1** Scheme of pathological events accompanying SCI and the therapeutic effects of MSCs on its pathobiology. BBB: blood–brain barrier; Ca: calcium; ROS: reactive oxygen species; NO nitric oxide; AA: amino acids; iNOS: inducible nitric oxide synthase; VEGF: vascular endothelial growth factor; BDNF: brain-derived neurotrophic factor; NGF: nerve growth factor; GDNF: glial cell-derived neurotrophic factor; IGF-1: insulin-like growth factor 1; ECM: extracellular matrix.

sprouting across the lesion [6]. Additionally, the activation of oligodendrocytes leads to the synthesis of oligodenrocyte-myelin glycoprotein and myelin-associated glycoprotein, both of which have neurite growth inhibitory activity [7–9].

As a result, the nonpermissive environment in the lesion prevents axons from regenerating and crossing the lesion cavity. Any novel therapeutic strategy should confront these obstacles by creating an anti-inflammatory environment that minimizes the glial scar component and provides neuroprotective support for the remaining host cells. It should also stimulate regeneration of the adult CNS and improve neurological functions. Cell therapy has the potential to enhance the quality of life of SCI patients.

## 49.3 Cell therapy

Among many treatments being developed for SCI, the placement of different types of grafts embedded with cell populations into areas of damage has been one of the most commonly used

regenerative approaches, attracting scientists and physicians for the last 15 years [10]. Initially, stem/stromal cells were used mainly because of their differentiation potential. However, it has been shown that they are able to secrete a large repertoire of signaling molecules, including anti-inflammatory cytokines and growth factors, which may modulate the inhibitory environment of SCI while increasing trophic support to the resident cells [10–12]. Trophic factors in the CNS that regulate the survival of neurons include NGF, BDNF, ciliary neurotrophic factor, and GDNF.

In addition, stem/stromal cells can be pro-oligodendrogliogenic, reduce scar formation, and secrete various anti-apoptotic and proangiogenic factors. Different sources and types of cells, including neural progenitor cells derived from embryonic stem cells, induced pluripotent cells, glial restricted precursors, mesenchymal stromal cells, olfactory ensheathing glia (OEG), and Schwann cells have all been examined in preclinical studies and in some cases in clinical studies.

Neural stem cells (NSCs), as well as neural progenitor cells derived from embryonic stem cells or from induced pluripotent stem cells, were tested for their ability to facilitate nerve regeneration and restore the neuronal circuitry when integrated into the injured site [13–15]. It was shown, that neural precursors of human origin can survive in the lesion, express neurotrophins, and facilitate the sprouting of endogenous axons. All of these actions lead to rapid improvements in locomotor functions and to the sparing of white and grey matter within 2–3 weeks of their transplantation. In the long term, transplanted neural precursor cells can slowly mature into region-specific mature neurons and can participate to some extent in tissue reconstruction [13,15].

OEGs are glial cells that ensheath olfactory axons within both the peripheral nervous system and the CNS portions of the primary olfactory pathway. They are responsible for the successful regeneration of olfactory axons throughout the life of adult mammals [16]. OEGs can create a permissive environment for axonal regeneration in the hostile environment of an SCI, supporting axonal growth and remyelination via the secretion of NGF, BDNF, neuregulins, and GDNF [17].

## 49.3.1 Mesenchymal stromal cells

Among cell populations tested, mesenchymal stromal cells (MSCs) have drawn the attention of many research groups. MSCs are self-renewing multipotent progenitor cells with the ability to differentiate into several mesenchymal phenotypes. Although they are not neuroectodermal in origin, and therefore do not normally differentiate into cells of neural lineages, they have been explored for the treatment of SCI. These multipotent adult stem cells are present in many tissues, including bone marrow, fat, placenta, umbilical cord (UC), UC blood, dental pulp, chorionic villi, amniotic membrane and fluid, and the spinal ligament, the ligamentum flavum. The sources that have been explored most commonly for SCI treatment are the bone marrow, adipose tissue, and the UC.

### 49.3.1.1 Bone-marrow-derived mesenchymal stromal cells

Traditionally, MSCs have been isolated from bone marrow even though bone marrow MSCs (BM-MSCs) have been shown to represent less than 1% of marrow mononuclear cells. Isolation of MSCs from the bone marrow is primarily by adhesion to plastic, but these cells can also be concentrated using Percoll gradient centrifugation.

BM-MSCs express a number of phenotypic markers. However, none of them are specific to these cells. According to the International Society for Cellular Therapy, human BM-MSCs under standard culture conditions must meet at least three criteria: (1) they must be plastic-adherent; (2) they must express C105, CD73, and CD90 and not CD45, CD34, CD14, CD11b, CD79 or CD19, or human leukocyte antigen (HLA)-DR surface molecules using flow cytometry; and (3) they must be capable of differentiating into osteoblasts, adipocytes, and chondroblasts *in vitro* [18]. Other markers that are generally accepted to be present on the surface of MSCs include CD44, CD71, stromal precursor antigen (Stro)-1 and adhesion molecules such as CD 106, CD166, and CD29 [19]. Human MSCs express class I major histocompatibility complex (MHC) antigens, HLA-A, -B and -C, but not MHC class II antigens such as HLA-DR as indicated above. They also lack the costimulatory molecules CD40, CD80, and CD86 [20].

Oliveri *et al.* performed a meta-analysis of the use of MSCs in animal models of SCI. Bone marrow was the most frequently used MSC tissue source (84%) and human MSCs were used in 36% of the studies. The majority of studies used the thoracic region as the site of induction of the SCI lesion [21], while only a few studies utilized cervical spinal cord lesions. In one study, BM-MSCs were injected into the lateral funiculus at 1 mm rostral and caudal to the lesion. The lateral funiculus is comprised of bundles of the anterior nerve roots emanating from the spinal cord and transmits nerve messages from the contralateral corticospinal and spinothalamic tracts. Grafted cells promoted extensive ingrowth of serotonin-positive raphespinal axons in the raphespinal tract, which is a tract of the spinal cord located in the lateral funiculus. The cells also promoted the growth of calcitonin-gene-related peptide (CGRP)-positive dorsal root sensory axons into the trauma zone, and significantly attenuated astroglial and microglial cell reactions. However, they also induced aberrant sprouting of CGRP-immunoreactive axons in Rexed's lamina III [22]. The Rexed laminae comprise a system of 10 layers of grey matter that label portions of the grey columns of the spinal cord.

Bone marrow contains different subpopulations of cells. Therefore, Urdzikova *et al.* compared the effect of MSCs with that of cells in the bone marrow mononuclear fraction as well as that of granulocyte colony-stimulating-factor-stimulated mobilized bone marrow cells. All three cell populations reduced locomotor deficits and cavity formation and spared white matter, regardless of the type of treatment [23]. Nearly all studies reporting on the use of BM-MSCs in SCI have observed such locomotor function improvement, although different

mechanisms of action have been put forward. Most often the effect was thought to be mediated by the release of growth factors, facilitating recovery by remyelinating spared white matter tracts, enhancing axonal growth, or by neuroprotective effects via the sparing of white and grey matter tissue in the lesion or by the downregulation of caspase-3-mediated apoptosis (Figure 49.1). Studies from Neuhuber's group reported a reduction of the acute inflammatory response to SCI, as well as a reduction of the cavity formation together with attenuation of microglia and macrophage reactivity [24,25]. Similarly, Hofstetter *et al.* observed not only less acute inflammation, but also the formation of guiding strands through the lesion [26]. BM-MSCs, when applied without *in vitro* predifferentiation, usually do not differentiate into either astrocytes or neurons. However, they can reduce lesion volume without altering astroglial or microglial activation [27]. BM-MSCs transplanted into rats with contusion lesions to the spinal cord exhibited significant improvements in motor and sensorimotor functions, which were strongly correlated with spared tissue volumes. Retrograde tracing revealed that rats given BM-MSCs had twice as many descending brainstem neurons with an axon projecting beyond the contused spinal cord segment. This correlated strongly with improved motor/sensorimotor functions but not with sensory functions [28]. Electrophysiological recordings showed that the latency of somatosensory evoked potentials and motor evoked potentials in MSC recipients were shorter than those of control animals [29,30].

The delivery route of cells is an important factor in the translation to clinical practice. Several studies have compared the intraspinal, intrathecal (or intracisternal), intra-arterial, and intravenous administration of BM-MSCs. Regardless of the delivery route, BM-MSC administration improved functional recovery after SCI. While engraftment of the transplanted cells was higher in animals given MSCs by the intraspinal route [29,31], injections into the parenchyma carry a risk of further damage to the spinal cord tissue; therefore, less invasive routes, such as intrathecal or intravenous delivery, are preferable. Cells administered via lumbar puncture, or intracisternally, showed the best functional recovery, even though the cells often remained in the intrathecal space [32,33] or accumulated around the anterior spinal artery [34]. Rats administered human BM-MSCs intrathecally had reduced inflammatory reactions and apoptosis, improved functional recovery, and rearranged glial scar formation after SCI, although no cells were detected in the spinal cord parenchyma 2 months after transplantation [33]. Repeated delivery of BM-MSCs may prolong the beneficial effects [34].

#### 49.3.1.2 Adipose-tissue-derived stromal cells

Adipose tissue is abundant in the body of most people. It is discarded after liposuction, and centrifugation of the lipoaspirate can isolate a stromal vascular fraction that is rich in stromal progenitor cells and endothelial cells as well as adipocytes. The stromal (mesenchymal) progenitor cells can differentiate *in vitro* into osteogenic, chondrogenic, and adipogenic cells. The *in vitro* and *in vivo* properties of adipose-tissue-derived stromal cells (ADSCs) are similar, although not identical, to those of BM-MSCs [35]. ADSCs in the stromal vascular fraction are identified phenotypically as follows: $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$, and $CD34^+$. The colony-forming unit assay for fibroblasts permits the evaluation of progenitor frequency in the stromal vascular fraction population. In culture, ADSCs retain markers in common with other MSC populations, including CD90, CD73, CD105, and CD44 and remain negative for CD45 and CD31. They can be distinguished from BM-MSCs by their positivity for CD36 and negativity for CD106 [36]. In models of SCI, ADSCs provide functional benefits by mechanisms similar to those of BM-MSCs, influencing angiogenesis, axonal outgrowth, inflammation, and gliosis. In one study, three-dimensional cell clusters of human ADSCs were transplanted into an SCI model [37]. The three-dimensional ADSCs differentiated *in vivo* into $CD31^+$ endothelial cells and remained differentiated. The grafted cells significantly elevated the density of vessel formation through angiogenic factors released at the site of the lesion and enhanced axonal outgrowth at the lesion site.

After direct delivery of human ADSCs in a rat spinal contusion model, abundant depositions of laminin of human origin were detected at the lesion site. Laminins are fibrous proteins present in the basal lamina of epithelia, one of the layers of the basement membrane. In neural development, laminin-111 is a major substrate along which nerve axons grow, both *in vivo* and *in vitro*. At the lesion site of rats given human ADSCs, clusters of neural precursors and blood vessels were found. Thus, laminin can be considered a factor mediating the proregenerative effect of human ADSCs in this animal model of SCI [38]. In experiments directly comparing the effect of direct injection of BM-MSCs and ADSCs in SCI models, the ADSC recipients had a significantly increased level of BDNF, which was associated with increased angiogenesis, preservation of axons, a decreased number of ED1 macrophages, and reduced cavity formation. These changes were accompanied with improved functional recovery [39]. (ED1 is a cellular marker specific for activated rat microglia, monocytes and macrophages).

#### 49.3.1.3 Umbilical cord mesenchymal stromal cells

Wharton's jelly is the mucoid connective tissue surrounding the two arteries and vein of the UC. It is composed of myofibroblast-like stromal cells, collagen fibers, and mucopolysaccharides, including hyaluronic acid and chondroitin sulfate. Human UCMSCs share many characteristics with BM-MSCs, including fibroblastic morphology, characteristic cell surface markers, cell cycle status, adipogenic and osteogenic differentiation capacity *in vitro*, secretion of a wide range of proteins, and support of hematopoiesis [40]. Several studies, however, have shown some differences between the two populations. The frequency of colony-forming units was significantly higher in UC-derived nucleated cells than in bone-marrow-derived nucleated cells, suggesting a higher frequency of MSCs in Wharton's jelly

compared with bone marrow. In addition, human UCMSCs have a faster doubling time than human BM-MSCs. This proliferative characteristic did not change after 30 passages [40]. There are also several potentially clinically important advantages of the use of UCMSCs. Unlike BM-MSCs, UCMSCs can undergo repeated freeze–thaw cycles without significant loss of viability or accumulating karyotypic abnormalities [41]. UCMSCs are readily obtainable after birth with no ethical constraints and can be reliably isolated. Like BM-MSCs, they can be engineered to express exogenous proteins. They may be less immunogenic than BM-MSCs. UCMSCs secrete a wide range of immunomodulatory and trophic factors known to promote neural cell survival, including fibroblast growth factor (FGF)2 and stromal cell-derived factor-1α, and neural differentiation (GDNF, BDNF, and NGF), making them a useful tool for neural rescue and support [3]. In a complete spinal cord transection model, transplanted UCMSCs survived for 16 weeks and produced large amounts of human neutrophil-activating protein-2, neurotrophin-3, basic FGF, glucocorticoid-induced tumor necrosis factor receptor, and VEGF receptor 3 [42]. Park and colleagues examined the potential effect of UCMSCs on endogenous cell proliferation and oligodendrogenesis after SCI. UCMSCs were transplanted into the boundary zone of the injured rat spinal cord. Newly synthesized ependymal and periependymal cells, which immunohistochemically resembled stem/progenitor cells because of their expression of neural/glial antigen 2, 2′,3′-cyclic-nucleotide 3′-phosphodiesterase (also known as CNPase), myelin basic protein, and glial fibrillary acid protein, were detected in the spinal cord tissue of grafted rats. CNPase is expressed exclusively by oligodendrocytes in the CNS, and the appearance of CNPase seems to be one of the earliest events of oligodendrocyte differentiation. CNPase is thought to play a critical role in the events leading up to myelination. (Ependyma is the thin epithelium-like lining of the ventricular system of the brain and the central canal of the spinal cord.) Behavioral analysis in this model showed that locomotor functions of the UCMSC group were significantly restored, the spinal cavity volumes were smaller, and the number of $TUNEL^+$ (apoptotic) cells was lower than in the control group [43] (TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling). When canine UCMSCs were compared with BM-MSCs and ADSCs in the same experiment, the application of UCMSCs led to more nerve regeneration, neuroprotection, and less inflammation compared with the other populations of MSCs [43].

#### 49.3.1.4 Neural differentiation of mesenchymal stromal cells

Several attempts have been made to transdifferentiate BM-MSCs or UCMSCs into neuron-like cells, oligodendrocytes, or Schwann cells. Many differentiation protocols have been published [44,45]. However, detailed examination of the protocols revealed that the morphological and immunocytochemical changes of MSCs following treatment with a neuronal induction and differentiation medium were not the result of neuronal differentiation, but represented cellular responses to chemical stress [46]. Neuronal differentiation of MSCs would require not only the adoption of neuronal morphology and gene expression, but also the acquisition of the electrophysiological properties of neurons. Experiments with β-mercaptoethanol-induced MSCs have shown that the neuron-resembling cells lacked action potentials and voltage-gated sodium and potassium currents 48 h after induction. The ability of MSCs to differentiate into cells of all three germ layers must be carefully examined as they have been reported to spontaneously express neural markers even in the undifferentiated state [47]. Electrophysiological evidence is essential for proving full differentiation to neuronal lineages.

#### 49.3.1.5 Pretreatment of mesenchymal stromal cells

In an attempt to enhance the therapeutic effectiveness of MSCs transplanted into rats with an acute SCI, several methods of pretreating the MSCs have been employed (Figure 49.2).

BM-MSCs were pretreated with valproic acid (VPA) before transplantation and then injected intravenously 1 week after SCI [48]. In animals, VPA raises cerebral and cerebellar levels of the inhibitory synaptic neurotransmitter γ-aminobutyric acid. This study demonstrated that pretreatment with VPA before transplantation enhanced the benefits of BM-MSCs in terms of greater cell migration and better neurological outcomes after traumatic SCI. The mechanism of this enhancement may have been related to an influence on the stromal cell-derived factor-1/CXCR4 axis [48]. In another study BM-MSCs were exposed simultaneously to inhibitors of DNA methylation, histone deacetylation, and pharmacological agents that increased cyclic adenosine monophosphate levels in an attempt to induce neural differentiation. The transplanted human MSCs survived, differentiated, and significantly improved locomotor recovery of SCI rats. Transplantation also reduced the volume of the lesion cavity and the loss of white matter [49]. UCMSC-derived neurospheres

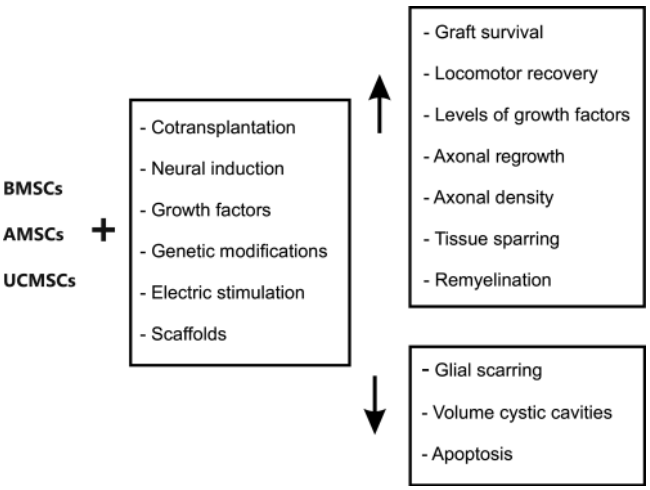



**Figure 49.2** Scheme showing modalities for enhancing MSC effects.

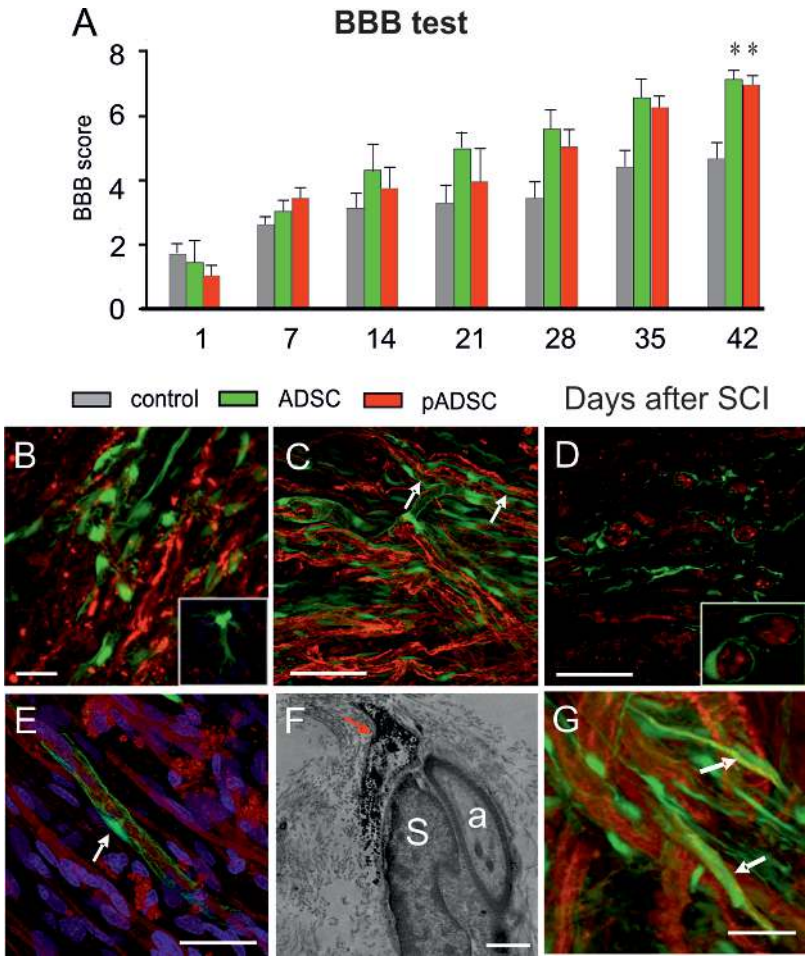



**Figure 49.3** Transplantation of ADSCs into SCI injury. (A) The Basso, Beattie, Bresnahan (BBB) test of rats with SCI grafted with predifferentiated (pADSCs) and naive ADSCs. Grafted animals showed better recovery regardless of the type of implanted cells. (B) Naive ADSCs did not form any interactions with host tissue 2 months after grafting into the SCI. (C, D) Predifferentiated pADSCs form tubules wrapping around the remaining filaments. (NF160, red; green fluorescent protein (GFP), green). (E) The myelinated fibers were covered (O1, red; GFP, green; 4′,6-diamidino-2-phenylindole, blue). (F) Transmission electron microphotograph showing a $GFP^+$ cell in close contact with a Schwann cell (S) and axon (a). (G) Transplanted cells expressed endothelial markers CD31 (CD31, red; GFP, green). Scale bar: B–D 20 μm; E, G, 25 μm; F, 1 μm. (See color plate section.) *Source:* adapted from Arboleda *et al.* 2011 [51], pp. 1113–1122.

supported by BDNF promoted morphologic and functional recovery in an animal model of transected spinal cord [50]. Treatment of BM-MSCs with the Schwann-cell-differentiating factors FGF, platelet-derived growth factor, and glial growth factor 2 significantly increased the production of BDNF *in vitro*.

Differentiated Schwann-like BM-MSCs transplanted into cervical spinal cord lesions remained at the injection sites, and in the trauma zone they were often associated with neurofilament-positive fibers and increased levels of VEGF. Differentiated BM-MSCs provided neuroprotection to axotomized rubrospinal neurons and increased the density of rubrospinal axons in the dorsolateral funiculus rostral to the injury site [22]. (Axotomy is the cutting or otherwise severing of the axon of a neuron. The rubrospinal tract is one of several major motor control pathways.) It is smaller and has fewer axons than the corticospinal tract, suggesting that it is less important in motor control. It is one of the pathways for the mediation of voluntary movement. The tract is responsible for large muscle movement as well as fine motor control.

As with BM-MSCs, ADSCs have also been also the subject of predifferentiation. ADSCs were predifferentiated into spheres prior to transplantation and their behavior was compared *in vivo* with naive ADSCs after transplantation into an SCI site. Both studies observed increased survival of predifferentiated ADSCs [51,52] (Figure 49.3). However, functional improvements were comparable in both groups, indicating that repair was mediated mainly through paracrine mechanisms.

#### 49.3.1.6 Co-transplantation

Owing to their anti-inflammatory and immunomodulatory effects, as well as their secretion of several neurotrophic factors, MSCs have been used as a cellular source of trophic support for endogenous and co-transplanted cells. The viability of mouse NSCs when they were co-transplanted with ADSCs was examined [53]. It was found that ADSCs preconditioned with hypoxia inhibited the apoptosis of murine NSCs in several *in vitro* injury models. Consistent with these *in vitro* results, murine NSCs transplanted into rat spinal cords with ADSCs showed better survival rates than murine NSCs transplanted alone [53]. Co-transplantation of Schwann cells and BM-MSCs promoted functional recovery in rat hind limbs, reduced the extent of the glial scar, and remyelinated the injured axons to a greater degree than single cell treatments and controls [54]. However, Amemori *et al.* co-transplanted OEGs and BM-MSCs and did

not observe a synergistic effect in the treatment of balloon-induced spinal cord compression [29].

#### 49.3.1.7 Mesenchymal stromal cells, growth factors, and neuroprotective factors

In order to promote survival of transplanted MSCs, the cells can be pretreated in culture prior to transplantation, or a neuroprotective agent can be administered at the same time as the cells. Salvianolic acid can alleviate oxidative stress and improve endothelial function. Salvianolic acid applied intraperitoneally with transplanted BM-MSCs provided neuroprotection to SCI and promoted the survival of the MSCs transplanted into the SCI [55]. Immunosuppression with the calcineurin inhibitor tacrolimus (originally known as FK506) also improved the survival of transplanted BM-MSCs [56]. Immortalized human BM-MSCs and neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) had synergistic protective effects that increased the number of remaining neuronal fibers in the injured spinal cord, leading to better locomotor functional recovery in SCI rats compared with administration of PACAP or human BM-MSCs alone [57].

Although MSCs secrete a large number of proteins, the level of these proteins is not sufficient for SCI repair. Poly(lactic-co-glycolic acid) (PLGA) is a copolymer that is used in a large number of therapeutic devices approved by the US Food and Drug Administration because of its biodegradability and biocompatibility. The sustained long-term release of FGF-2 from heparin-conjugated PLGA-conjugated nanospheres co-transplantated with BM-MSCs following rat SCI led to increased functional improvement, greater survival of the human BM-MSCs, and a greater density of neurofilament-positive cells in the center of the injury [58].

Animals co-transplanted with human UCMSC-derived neurospheres (HUMSC-NSs) and BDNF had greatly improved BBB test scores compared with HUMSC-NSs-treated and vehicle-treated control groups. The BBB scoring system was devised by Drs Basso, Beattie, and Bresnahan in 1995 in order to study the sequence of locomotor recovery patterns. It takes into consideration the early (BBB score from 0 to 7), intermediate (8–13), and late phases (14–21) of recovery. Any treatment which increases the BBB score may be considered neuroprotective in animal models. Additionally, axonal regeneration showed significant improvement in rats receiving HUMSC-NSs and BDNF compared with HUMSC-NSs-treated and medium-treated groups as demonstrated by staining for the 200 kDa neurofilament protein NF-200$^{+}$ and fluorogold retrograde tracing [50]. The retrograde fluorescent tracer fluorogold is useful, first, because it is not only detectable by fluorescence microscopy but also immunocytochemically, resulting in an almost complete staining of the dendritic arbor. Second, it is visible in lysosome-like structures allowing for the identification of projection neurons at the ultrastructural level and, third, it remains in the labeled neurons for extended periods of time.

Genetically modified neurotrophin 3 (NT-3) UCMSCs were transplanted following a clip injury to the rat spinal cord. A clip injury is produced by clipping the spinal cord extradurally with a modified aneurysm clip. NT-3-UCMSCs recipients achieved better functional recovery with an increased intensity of 5-hydoxytryptamine (serotonin) fibers, increased volume of spared myelination, and decreased area of the cystic cavity in the NT-3-UCMSCs group compared with the UCMSCs group. Moreover, transplanted NT-3-UCMSCs survived and produced larger amounts of NT-3 than the UCMSCs in the spinal cord of the recipients [59].

Zeng and colleagues have performed several studies with BM-MSCs overexpressing tropomyosin receptor kinase C (TrkC), indicating that TrkC may play a role in BM-MSC homing [60,61]. TrkC is also known as NT-3 growth factor receptor, neurotrophic tyrosine kinase receptor type 3 or TrkC tyrosine kinase. TrkC is the high-affinity receptor for NT-3. As such, TrkC mediates the multiple effects of this neurotrophic factor, which include neuronal differentiation and survival.

Rats with combined treatments comprising of platelet-rich plasma and BDNF-overexpressing BM-MSC implants showed enhanced hind limb locomotor performance with more axonal remyelination and less accumulation of astrocytes at the transplant site at 8 weeks compared with control animals [62].

MSCs that overexpressed basic FGF produced enhanced myelination, axonal regeneration in the lesion, and recovery of neurological function after SCI in rats [63].

Hepatocyte growth factor (HGF) has antiglial scar effects. Transplantation of HGF-overexpressing BM-MSCs (HGF-BM-MSCs) into the cervical hemisection reduced scar formation. Animals treated with HGF-BM-MSCs showed axonal growth promotion beyond the glial scars and improved recovery of forepaw function [64].

#### 49.3.1.8 Mesenchymal stromal cells and external physical forces

Electrical stimulation has been reported to promote the survival and differentiation of the BM-MSCs. The number and ratio of BM-MSCs surviving near the lesion site were significantly increased in the BM-MSC and electrical stimulation group compared with groups treated with either BM-MSCs alone or electrical stimulation alone. This suggested that the implanted spike wave evoked by electrical stimulation could promote the bioactivity of BM-MSCs as well as their survival [65]. Zeng and colleagues reported in several studies that electric acupuncture promoted the differentiation of BM-MSCs into neuron-like and oligodendrocyte-like cells as well as the regeneration of 5-hydroxytryptamine (serotonin)-positive nerve fibers in injured spinal cords via the induction of endogenous NT-3. A combination of electric acupuncture and BM-MSC transplantation can improve the partial functionality of paralyzed hind limbs in transected rats [61,66,67].

The efficiency of cell delivery in SCI can be increased using magnetic systems with tunable geometric parameters. The effects

of a pulsed electromagnetic field (PEMF) on injured rat spinal cords were investigated using human MSCs into which magnetic nanoparticles had been incorporated. The MSCs were guided by the PEMF toward the injured site; following exposure for 8 h each day over a period of 4 weeks, behavioral recovery occurred in SCI rats, suggesting that PEMF enhanced the action of the transplanted cells [68].

Vanecek *et al.* showed that intrathecally transplanted cells labeled with superparamagnetic iron oxide (SPIO) nanoparticles could be guided by a magnetic field and successfully targeted to a position near the lesion site in the rat spinal cord. Cell distribution correlated well with the calculated distribution of magnetic forces exerted on the transplanted cells in the subarachnoid space and lesion site [69].

To follow the migration and fate of transplanted cells, an SPIO labeling technique was successfully used with *in vivo* magnetic resonance imaging (MRI) in stroke or SCI models [23,70]. MRI cell detection *in vivo* was confirmed by histologically using iron staining or fluorescent cellular markers that were be coupled to SPIO nanoparticles [71].

#### 49.3.1.9 Mesenchymal stromal cells and scaffolds

The standard method of MSC delivery for SCI is intraspinal injection, but this can result in additional injury and provides little control over how the cells integrate into the tissue. Moreover, in the case of large SCI or chronic spinal cord lesion, when cystic cavities are common, it is necessary to reconstruct the damaged tissue with a growth-promoting environment that can bridge the lesion and stimulate host tissue regeneration. One approach is to fill the cavity with a bioengineered scaffold that can provide structural and/or chemical support for axonal regrowth and concomitantly serve as a matrix for the delivery of cells or drugs and bioactive substances [72,73]. Scaffolds are available for the treatment of subacute and chronic lesions. Poly(2-hydroxyethyl methacrylate) (PHEMA) is a polymer that forms a hydrogel in water. Delayed implantation of positively charged PHEMA hydrogels 1 week after a spinal cord transection was shown to be more effective than immediate hydrogel implantation, as it significantly reduced cavity volume [74].

The survival time of BM-MSCs or UCMSCs was assessed by grafting acellular spinal cord scaffolds seeded with BM-MSCs or UCMSCs in a rat model of acute hemisected SCI. Spinal cord scaffolds seeded with MSCs enhanced grafted cell survival, improved differentiation, promoted axonal regeneration at the site of the lesion, promoted migration of host oligodendrocytes into the graft, and reduced endogenous nerve tissue apoptosis, thus protecting the host tissue as well as improving functional recovery from the implant [75,76].

MSC survival was improved 6 months after the implantation of poly[*N*-(2-hydroxypropyl) methacrylamide with the attached amino acid sequence Arg–Gly–Asp] (HPMA-RGD) that had been seeded with MSCs. A cell polymer scaffold bridged the lesion, reduced atrophy, and facilitated the ingrowth of axons, vessels, Schwann cells, and astrocytes into the lesion [77].

PLGA scaffolds seeded with human BM-MSCs were implanted into a completely transected spinal cord. BM-MSCs were detected at the implanted site 4 and 8 weeks after transplantation, indicating *in vivo* survival of implanted human BM-MSCs. Immunostained images showed clear evidence of axonal regeneration in human BM-MSC-seeded PLGA scaffolds only, and this resulted in functional recovery of a completely transected SCI [78].

## 49.4 Chronic spinal cord injury

Although the acute and subacute phases of SCI have been extensively studied, there have only been a limited number of preclinical studies in chronic animal models; as a result, it is more difficult to evaluate the efficacy of an MSC-based approach. Studies reported to date have not been as encouraging as studies performed in the acute or subacute phases of SCI. The effects of BM-MSCs alone was compared with that of BM-MSCs and the immunosuppressive agent tacrolimus. The findings indicated that immunosuppression improved the survival of the grafted cells. A cell-dose effect was found regarding locomotion recovery and tissue protection independent of immunosuppression. Nevertheless, immunosuppression enhanced the electrophysiological outcomes and allowed filling of the cavity formed after injury by new regenerative tissue and axons [56].

Highly purified Stro-1-positive human mesenchymal precursor cells were assessed for their ability to repair injured spinal cord at 3 or 6 weeks after the SCI [79]. Behavioral and anatomical analysis showed a statistically significant improvement in functional recovery, tissue sparing, and cyst volume reduction following cell therapy. This was due to an increased presence of serotonergic and sensory (CGRP) axons. Zurita *et al.* observed progressive functional recovery 3 months after SCI in paraplegic pigs that had been injected with autologous BM-MSCs suspended in autologous plasma into the lesion zone and adjacent subarachnoid space. Intramedullary cavities caused by the injury were filled with a newly formed tissue containing several axons together with surviving BM-MSCs. In the treated animals, recovery of the previously abolished somatosensory-evoked potentials was detected [80].

NT-3 BM-MSCs were injected into the lesion site of rats with cervical SCI 6 weeks after induction of the lesion [81]. A modest number of axons penetrated through the chronic scar that contained a mixture of inhibitory and growth-stimulating factors.

Behavioral analysis showed a statistically significant improvement in rats implanted with HPMA-RGD hydrogels seeded with BM-MSCs 5 weeks after SCI. The combined approach prevented tissue atrophy, and the hydrogels were infiltrated with axons myelinated with Schwann cells, blood vessels and astrocytes [77] (Figure 49.4).

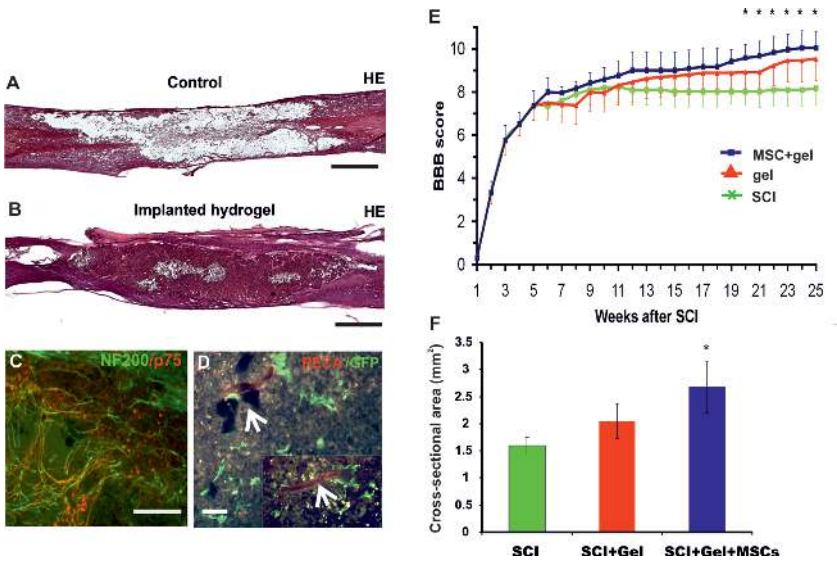



**Figure 49.4** Hydrogel scaffold seeded with BM-MSCs implanted into a chronic lesion 6 months after SCI. (A) A cavity developed at the lesion site 6 months after induction of the lesion. (B) The implanted hydrogel adhered well to the surrounding tissue and completely bridged the posttraumatic cavity. (C) The porous structure of the hydrogel was completely infiltrated with axons ($NF200^+$) and Schwann cells ($p75^+$). (D) Transplanted cells survived for 6 months in the hydrogel. Blood vessels stained with rat endothelial cell antigen (RECA) infiltrated the hydrogel. (E) Animals transplanted with the hydrogels seeded with cells showed improved in locomotor activity. (F) Tissue atrophy was reduced in animals with a cell–polymer construct. Scale bar: A, B: 500 µm; C: 50 µm; D: 15 µm. (See color plate section.) *Source*: adapted from Hejcl 2010 [77], pp. 1535–1546.

## 49.5 Cellular transplants for spinal cord injury

The annual total incidence of SCI ranges from 12 to 71 per million people in different populations, and approximately $700 \pm 500$ per million patients are living with SCI [82–86]. The most frequent causes of SCI are motor vehicle accidents, falls, and acts of violence. SCI has been considered an untreatable condition for a very long time. Initial references to it come from the Edwin Smith papyrus (Figure 49.5). This document is an Ancient Egyptian medical text, named after the dealer who bought it in 1862, and is the oldest known surgical treatise on trauma. This document, which may have been a manual of military surgery, describes 48 cases of injuries, fractures, wounds, dislocations, and tumors. It dates to Dynasties 16–17 of the Second Intermediate Period in Ancient Egypt, *circa* 1500 BC.



**Figure 49.5** Plates VI and VII of the Edwin Smith Papyrus at the Rare Book Room, New York Academy of Medicine. *Source:* https://commons.wikimedia.org/wiki/File:Edwin_Smith_Papyrus_v2.jpg#/media/File:Edwin_Smith_Papyrus_v2.jpg.

The document described two cases of SCI and determined them as "an ailment not to be treated" [87]. The idea that axons have an intrinsic ability to regrow comes from the work of Ramon y Cajal in the 1920s, who described how the capacity to regenerate is inhibited by scar tissue formation at the site of injury. Despite a long history of spinal cord regeneration research, the progress of therapeutic intervention has been limited.

Current management of SCI is based on two multicenter controlled studies, the STASCIS [88] and NASCIS trials [89] that advocated early decompression and corticosteroid therapy respectively. Both clinical trials have engendered controversies. However, these are more related to the framework of the clinical trials as opposed to the treatments advocated [90]. Despite the modest therapeutic approaches available, 679 clinical trials related to SCI have been registered at clinicaltrials.gov. The trials are mostly related to rehabilitation, electrostimulation, pharmacological repair strategies, surgical management, cell therapies, and treatments of accompanying diseases such as pain management or hypertension. Rehabilitation after SCI plays an increasingly important role in recovery. Its primary goals are the prevention of secondary complications, maximization of physical functioning, and reintegration of the patient into the community.

### 49.5.1 Use of hematopoietic stem/progenitor cells for spinal cord injury

The rationale for cellular transplants as a strategy for SCI is based on the idea that living cells will respond better than a drug to the wide spectrum of pathological events that occur after injury. The proposed mechanisms include minimalization of cell loss

through immunomodulation, neuroprotection through trophic support, tissue modification, and cell replacement. Cells can be administered effectively by lumbar puncture into the cerebrospinal fluid; thus, no additional surgical intervention is required and there is the possibility of repeated applications. Selective intra-arterial and intravenous administrations are other routes that have been used in clinical studies to treat SCI. The first clinical trials in SCI patients based on cell therapy used an unmanipulated mononuclear cell fraction isolated from bone marrow (BMCs) or sorted $CD34^+$ hematopoietic stem cells isolated from mobilized peripheral blood. We compared intra-arterial administration (using the vertebral artery, $n = 6$) versus intravenous administration of bone marrow nucleated cells ($n = 14$) in a group of subacute (10–33 days after SCI, $n = 8$) and chronic patients (2–18 months after SCI, $n = 12$) [91]. Motor evoked potentials, sensory evoked potentials, MRI, and the American Spinal Injury Association (ASIA) scores were used for monitoring patient follow-up. The ASIA scoring system is a system of tests used to define and describe the extent and severity of a patient's SCI and help determine future rehabilitation and recovery needs. It is ideally completed within 72 h after the initial injury. The patient's grade is based on how much sensation they can feel at multiple points on the body as well as on tests of motor function. We found that the administration of bone marrow mononuclear cells after SCI was safe. A partial improvement in the ASIA score and partial recovery of motor evoked potentials or sensory evoked potentials were observed in all subacute patients who received the cells via the vertebral artery ($n = 4$) and in one of four patients with subacute SCI who received the cells intravenously. Improvement also occurred in one patient with chronic SCI who received cells via the vertebral artery [91].

Park *et al.* evaluated the therapeutic efficacy of autologous bone marrow cells implanted at the site of the SCI in combination with granulocyte-macrophage colony-stimulating factor (GM-CSF) given by subcutaneous injection [92]. No serious side effects were observed. There were improvements in the ASIA score, and MRI 4–6 months after injury showed a slight improvement at the site of cell injection [92].

Yoon *et al.* implanted autologous bone marrow cells directly into the spinal cord and reported that neurological improvement occurred in acute and subacute SCI patients, but not in those in the chronic period [93].

The study performed by Callera and do Nascimento demonstrated the feasibility and safety of delivering autologous bone marrow mononuclear cells after GM-CSF administration via lumbar puncture to patients with SCI. Interestingly, there was an absence of cells in cerebrospinal fluid samples obtained 7 days after their administration [94], an observation made also in rodent models of SCI [33]. Similarly, Kumar *et al.* injected autologous bone marrow cells by lumbar puncture and reported improvements in neurological function in one-third of SCI patients [95]. The time elapsed between injury and the administration of cells and the number of $CD34^+$ cells injected influenced the outcome of the approach. Deda *et al.* administered autologous hematopoietic stem/progenitor cells cells to nine chronic patients and concluded that their application was safe regardless of the route of administration (intrathecally, directly into the cord, and intravenously) [96]. Geffner *et al.* administered autologous bone marrow stem cells directly into the spinal cord, into the spinal canal, and intravenously [97].

### 49.5.2 Use of mesenchymal stromal cells for spinal cord injury

BM-MSCs have been used in a number of studies. Kishk *et al.* administered MSCs intrathecally monthly within 6 months of injury [98]. Dai *et al* administered MSCs intrathecally [99]. Each reported improvements after BM-MSC administration.

Karamouzian *et al.* reported a marked recovery in three patients after intrathecal application of MSCs but the results were statistically borderline [100]. Pal *et al.* did not find any neurological or electrophysiological improvements after BM-MSC administration [101]. Bhanot *et al.* used autologous MSCs to treat chronic SCI that were administrated directly to the injury site. They concluded that the administration of such cells was safe, but may not have been efficacious [102].

Ra *et al.* administered adipose-tissue-derived MSCs intravenously to eight male patients who had had an SCI >12 months previously. No serious adverse events related to administration of the cells during the 3-month follow-up period were reported [103].

Clinical studies involving MSCs derived from UC blood are still limited. Cheng *et al.* transplanted UCMSCs into the subarachnoid space by lumbar puncture and compared the outcome with that with rehabilitation therapy. They concluded that MSCs from the umbilical matrix improved recovery of neurological functional after SCI and that their efficacy was superior to that of rehabilitation therapy [104].

The safety and efficacy of umbilical MSCs administered by intrathecal injection have been reported by Liu *et al.* Treatment was effective in 13 of 22 patients and no treatment-related adverse events occurred during a follow-up period ranging from 3 months to 3 years [105]. All published clinical studies are summarized in Table 49.1.

## 49.6 Conclusions

The application of cells represents a promising approach in the treatment of SCI. From experimental studies in rats with traumatic SCI it is evident that MSCs applied during the subacute phase of SCI decrease the size of the lesion, prevent cavity formation, and have a substantial beneficial effect on locomotor recovery. There are several possible mechanisms underlying these effects, including the production of growth factors, a decrease in anti-inflammatory cytokines, glial scar remodeling, and support of revascularization. Their effects are observed mainly in the rescue of endogenous tissue and support of neuroregeneration, and can be enhanced by genetic engineering to

**Table 49.1** Published clinical trials using bone marrow mononuclear cells or mesenchymal stromal cells for the treatment patients with SCI.

| Authors | Number of patients in the study | Type of cells/routes | Follow-up period | Results | Complications |
|---|---|---|---|---|---|
| Park *et al.* [92] | 4 acute patients, 2 chronic patients | $1.1 \times 10^6$/μL autologous BMCs IS, GM-CSF SC for 5 days | 6–12 months | 1 × ASIA A–B, 4 × ASIA A–C | Fever, myalgia, leukocytosis after GM-CSF injections |
| Callera *et al.* [94] | 10 chronic patients | $2 \times 10^9$ BMCs IT | Not provided | No detailed assessment of the results | No complications |
| Syková *et al.* [91] | 7 acute/ subacute patients, 13 chronic patients | $10^{12}$ BMCs IA 6 patients; IV 14 patients | 3–12 months | IA: 4 patients ASIA improvement; IV: 1 patient ASIA improvement | No complications |
| Yoon *et al.* [93] | 17 acute patients, 6 subacute, 12 chronic patients | $2 \times 10^9$ BMCs IS, once daily for 5 days, GM-CSF SC | 10 months | 29.5% acute, 33.3% subacute, and no chronic patients improved | Fever, headache after GM-CSF application, neuropathic pain |
| Deda *et al.* [96] | 9 chronic patients | $10–67 \times 10^6$ autologous BMCs IS, IV into gel covering the spinal lesion | 1 year | 100% improvement 1 × A–B, 8 × A–C | No complications |
| Geffner *et al.* [97] | 4 acute, 4 chronic patients | $4 \times 10^8$ autologous BM-MSCs, repeated injections IT, IV, and IS | 2 years | Acute SCI: 3 × ASIA A–C Chronic SCI: 1 × ASIA A–C, 1 × B–C, 1 × C–D | No complications |
| Pal *et al.* [101] | 15 acute, 10 chronic | $10^6$ culture-expanded BM-MSCs, two doses IT | 1–3 years | No ASIA improvement | No complications |
| Hammadi *et al.* [106] | 277 chronic patients | $5 \times 10^8$ hematopoietic stem cells IT, repetitive 1–4 times after 6–8 weeks | 4 weeks | Improvement in 120 patients (43%); 88 × A–B, 32 × A–C 157 patients no improvement | 90% temporary back pain, meningism |
| Saito *et al.* [107] | 5 acute patients | $3–5 \times 10^7$ culture-expanded autologous BMSCs IT | 6 months | 3 × ASIA A 1 × ASIA B–D 1 × ASIA C–D | No complications |
| Kishk *et al.* [98] | 44 chronic patients | $5–10 \times 10^6$/kg BM-MSCs IT once per month for 6 doses | 18 months | 18 × improvement of motor score | Spasticity, neuropathic pain, myelitis, and encephalomyelitis in 1 patient |
| Park *et al.* [108] | 10 chronic patients | $8 \times 10^6$ culture-expanded BM-MSCs IS + $4 \times 10^7$ ID + $5 \times 10^7$ IT | 6–62 months | Improvement in strength tests in 3 patients; 7 patients no improvement | |
| Karamouzian *et al.* [100] | 11 acute/ subacute patients | From $7 \times 10^5$ to $1.2 \times 10^6$ BM-MSCs IT | 12–33 months | 5 patients improvement | No complications |
| Jiang *et al.* [109] | 20 chronic patients | $1 \times 10^8$ BM-MSCs IT, IL | 30 days | 75% of patients ASIA improvement | Fever, headache |
| Amr *et al.* [110] | 14 chronic patients | BM-MSCs in combination with chitosan–laminin-based scaffold and peripheral nerve grafts | 2 years | 14 × ASIA A–C, 2 × ASIA A–B | Seroma after degradation of chitosan, 1 × hematoma as a result of healing process |
| Moviglia *et al.* [111] | 8 chronic | BMCs IA; effector T cells IV; NSCs IA | 12 months | 5 × ASIA A–D | Fever, headache, and a tingling sensation |
| Kumar *et al.* [95] | 297 patients | Unmanipulated BMCs via lumbar puncture (IT) | 3 months | 32.6% of patients sensory and motor improvement | No complications |
| Cristante *et al.* [112] | 39 chronic patients | Autologous undifferentiated hematopoietic stem/ progenitor cells mobilized by GM-CSF IA | 2.5 years | 66.7% patients recovery of somatosensory evoked response | No complications from cell therapy |

**Table 49.1** (*Continued*)

| Authors | Number of patients in the study | Type of cells/routes | Follow-up period | Results | Complications |
|---|---|---|---|---|---|
| Dai *et al.* [99] | 40 chronic patients | Autologous BM-MSCs IS | 6 months | 10 patients improved in terms of motor, light touch, pin prick sensation, and residual urine volume | No complications |
| Ra *et al.* [103] | 8 chronic patients | ADSCs IV | 3 months | Cell treatment appeared safe; no tumor development | No complications |
| Cheng *et al.* [104] | 34 chronic patients | $2 \times 10^7$ double injections of UCMSCs IS | 6 months | 7 patients had significant and stable improvement in movement, self-care ability, and muscular tension | Radiating neuralgia |
| Liu *et al.* [105] | 22 patients | UCMSCs cells IT | 3 months–3 years | 13 patients improved in motor or sensory functions | Headache and low back pain |

IA: intra-arterially; ID: intradurally; IL: intralesionally; IS: intraspinally; IT: intrathecally; IV: intravenously; SC: subcutaneously.

oversecrete specific molecules. Unlike neural progenitors, MSCs do not differentiate; therefore, they cannot replace lost populations of neural cells. Unfortunately, patients with SCI often suffer from multiple injuries and consequently the application of cell therapy in the acute or subacute phase is not always possible. The consequent severe degeneration, cavity formation (syringomyelia), and atrophy of grey and white matter prevents regeneration. Bridging the defect with a suitable artificial or biological scaffold is therefore needed. Future approaches to chronic SCI treatment should focus on the development of multifaceted therapeutic approaches that would stimulate the regeneration process. These would comprise of scaffolds as cell carriers, enzymes that can digest glial scarring, growth factors to support cell survival and axonal regeneration, and blockers of molecules that adversely affect the SCI milieu. The optimal combination and timing of these processes may result in a synergistic effect in spinal cord treatment.

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## References

1 Priebe MM, Chiodo AE, Scelza *et al.* Spinal cord injury medicine. 6. Economic and societal issues in spinal cord injury. *Archives of Physical Medicine and Rehabilitation* 2007;**88**(3 Suppl 1): S84–S88.

2 Dasari V, Veeravalli KK, Rao JS, *et al.* Mesenchymal stem cell therapy for apoptosis after spinal cord injury. In: R. Chuen-ChungC Chang (ed.), *Advanced Understanding of Neurodegenerative Diseases*. InTech: Rijeka, 2011; pp. 365–394.

3 Vawda R, Fehlings MG. Mesenchymal cells in the treatment of spinal cord injury: current & future perspectives. *Current Stem Cell Research & Therapy* 2013;**8**(1):25–38.

4 Nathaniel EJ, Nathaniel DR. Astroglial response to degeneration of dorsal root fibers in adult rat spinal cord. *Experimental Neurology* 1977;**54**(1):60–76.

5 Eddleston M, Mucke L. Molecular profile of reactive astrocytes – implications for their role in neurologic disease. *Neuroscience* 1993;**54**(1):15–36.

6 Fawcett JW. The glial response to injury and its role in the inhibition of CNS repair. In: M. Bähr (ed.), *Brain Repair*. Advances in Experimental Medicine and Biology, vol. **557**. Springer, 2006; pp. 11–24.

7 Schwab ME, Caroni P. Oligodendrocytes and CNS myelin are nonpermissive substrates for neurite growth and fibroblast spreading *in vitro*. *Journal of Neuroscience* 1988;**8**(7): 2381–2393.

8 Oertle T, van der Haar ME, Bandtlow CE, *et al.* Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *Journal of Neuroscience* 2003;**23**(13):5393–5406.

9 Domeniconi M, Cao Z, Spencer T, *et al.* Myelin-associated glycoprotein interacts with the Nogo66 receptor to inhibit neurite outgrowth. *Neuron* 2002;**35**(2):283–290.

10 Tetzlaff W, Okon EB, Karimi-Abdolrezaee S, *et al.* A systematic review of cellular transplantation therapies for spinal cord injury. *Journal of Neurotrauma* 2011;**28**(8):1611–1682.

11 Lu P, Jones LL, Snyder EY, Tuszynski MH. Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury. *Experimental Neurology* 2003;**181**(2):115–129.

12 Meyerrose T, Olson S, Pontow S, *et al.* Mesenchymal stem cells for the sustained *in vivo* delivery of bioactive factors. *Advanced Drug Delivery Reviews* 2010;**62**(12):1167–1174.

13 Amemori T, Romanyuk N, Jendelova P, *et al.* Human conditionally immortalized neural stem cells improve locomotor function after

spinal cord injury in the rat. *Stem Cell Research & Therapy* 2013;**4**(3):68.

14 Erceg S, Ronaghi M, Oria M, *et al.* Transplanted oligodendrocytes and motoneuron progenitors generated from human embryonic stem cells promote locomotor recovery after spinal cord transection. *Stem Cells* 2010;**28**(9):1541–1549.

15 Romanyuk N, Amemori T, Turnovcova K, *et al.* Beneficial effect of human induced pluripotent stem cell-derived neural precursors in spinal cord injury repair. *Cell Transplantation* 2015;**24**(9):1781–1797.

16 Boyd JG, Doucette R, Kawaja MD. Defining the role of olfactory ensheathing cells in facilitating axon remyelination following damage to the spinal cord. *FASEB Journal* 2005;**19**(7):694–703.

17 Woodhall E, West AK, Chuah MI. Cultured olfactory ensheathing cells express nerve growth factor, brain-derived neurotrophic factor, glia cell line-derived neurotrophic factor and their receptors. *Brain Research. Molecular Brain Research* 2001;**88**(1–2):203–213.

18 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;**8**(4):315–317.

19 Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells* 2007;**25**(11):2739–2749.

20 Wong RS. Mesenchymal stem cells: angels or demons? *Journal of Biomedicine & Biotechnology* 2011;**2011**:459510.

21 Oliveri RS, Bello S, Biering-Sorensen F. Mesenchymal stem cells improve locomotor recovery in traumatic spinal cord injury: systematic review with meta-analyses of rat models. *Neurobiology of Disease* 2014;**62**:338–353.

22 Novikova LN, Brohlin M, Kingham PJ, *et al.* Neuroprotective and growth-promoting effects of bone marrow stromal cells after cervical spinal cord injury in adult rats. *Cytotherapy* 2011;**13**(7):873–887.

23 Urdzikova L, Jendelova P, Glogarova K, *et al.* Transplantation of bone marrow stem cells as well as mobilization by granulocyte-colony stimulating factor promotes recovery after spinal cord injury in rats. *Journal of Neurotrauma* 2006;**23**(9):1379–1391.

24 Himes BT, Neuhuber B, Coleman C, *et al.* Recovery of function following grafting of human bone marrow-derived stromal cells into the injured spinal cord. *Neurorehabilitation and Neural Repair* 2006;**20**(2):278–296.

25 Neuhuber B, Himes BT, Shumsky JS, *et al.* Axon growth and recovery of function supported by human bone marrow stromal cells in the injured spinal cord exhibit donor variations. *Brain Research* 2005;**1035**(1):73–85.

26 Hofstetter CP, Schwarz EJ, Hess D, *et al.* Marrow stromal cells form guiding strands in the injured spinal cord and promote recovery. *Proceedings of the National Academy of Sciences of the United States of America* 2002;**99**(4):2199–2204.

27 Boido M, Garbossa D, Fontanella M, *et al.* Mesenchymal stem cell transplantation reduces glial cyst and improves functional outcome after spinal cord compression. *World Neurosurgery* 2014;**81**(1):183–190.

28 Ritfeld GJ, Nandoe Tewarie RD, Vajn K, *et al.* Bone marrow stromal cell-mediated tissue sparing enhances functional repair after spinal cord contusion in adult rats. *Cell Transplantation* 2012;**21**(7):1561–1575.

29 Amemori T, Jendelova P, Ruzickova K, *et al.* Co-transplantation of olfactory ensheathing glia and mesenchymal stromal cells does not have synergistic effects after spinal cord injury in the rat. *Cytotherapy* 2010;**12**(2):212–225.

30 Lee KH, Suh-Kim H, Choi JS, *et al.* Human mesenchymal stem cell transplantation promotes functional recovery following acute spinal cord injury in rats. *Acta Neurobiologiae Experimentalis* 2007;**67**(1):13–22.

31 Kim JW, Ha KY, Molon JN, Kim YH. Bone marrow-derived mesenchymal stem cell transplantation for chronic spinal cord injury in rats: comparative study between intralesional and intravenous transplantation. *Spine* 2013;**38**(17):E1065–E1074.

32 Mothe AJ, Bozkurt G, Catapano J, *et al.* Intrathecal transplantation of stem cells by lumbar puncture for thoracic spinal cord injury in the rat. *Spinal Cord* 2011;**49**(9):967–973.

33 Urdzikova LM, Ruzicka J, LaBagnara M, *et al.* Human mesenchymal stem cells modulate inflammatory cytokines after spinal cord injury in rat. *International Journal of Molecular Sciences* 2014;**15**(7):11275–11293.

34 Cizkova D, Novotna I, Slovinska L, *et al.* Repetitive intrathecal catheter delivery of bone marrow mesenchymal stromal cells improves functional recovery in a rat model of contusive spinal cord injury. *Journal of Neurotrauma* 2011;**28**(9):1951–1961.

35 Danišovic L, Lesný P, Havlas V, *et al.* Chondrogenic differentiation of human bone marrow and adipose tissue-derived mesenchymal stem cells. *Journal of Applied Biomedicine* 2007;**5**:139–150.

36 Bourin P, Bunnell BA, Casteilla L, *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013;**15**(6):641–648.

37 Oh JS, Park IS, Kim KN, *et al.* Transplantation of an adipose stem cell cluster in a spinal cord injury. *Neuroreport* 2012;**23**(5):277–282.

38 Menezes K, Nascimento MA, Goncalves JP, *et al.* Human mesenchymal cells from adipose tissue deposit laminin and promote regeneration of injured spinal cord in rats. *PLoS ONE* 2014;**9**(5):e96020.

39 Zhou Z, Chen Y, Zhang H, *et al.* Comparison of mesenchymal stromal cells from human bone marrow and adipose tissue for the treatment of spinal cord injury. *Cytotherapy* 2013;**15**(4):434–448.

40 Cao FJ, Feng SQ. Human umbilical cord mesenchymal stem cells and the treatment of spinal cord injury. *Chinese Medical Journal* 2009;**122**(2):225–231.

41 Troyer DL, Weiss ML. Wharton's jelly-derived cells are a primitive stromal cell population. *Stem Cells* 2008;**26**(3):591–599.

42 Karahuseyinoglu S, Cinar O, Kilic E, *et al.* Biology of stem cells in human umbilical cord stroma: *in situ* and *in vitro* surveys. *Stem Cells* 2007;**25**(2):319–331.

43 Ryu HH, Kang BJ, Park SS, *et al.* Comparison of mesenchymal stem cells derived from fat, bone marrow, Wharton's jelly, and umbilical cord blood for treating spinal cord injuries in dogs. *The Journal of Veterinary Medical Science* 2012;**74**(12):1617–1630.

44 Sanchez-Ramos J, Song S, Cardozo-Pelaez F, *et al.* Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Experimental Neurology* 2000;**164**(2):247–256.

45 Woodbury D, Schwarz EJ, Prockop DJ, Black IB. Adult rat and human bone marrow stromal cells differentiate into neurons. *Journal of Neuroscience Research* 2000;**61**(4):364–370.

46 Lu P, Blesch A, Tuszynski MH. Induction of bone marrow stromal cells to neurons: differentiation, transdifferentiation, or artifact? *Journal of Neuroscience Research* 2004;**77**(2):174–191.

47 Deng J, Petersen BE, Steindler DA, *et al.* Mesenchymal stem cells spontaneously express neural proteins in culture and are neurogenic after transplantation. *Stem Cells* 2006;**24**(4): 1054–1064.

48 Chen L, Cui X, Wu Z, *et al.* Transplantation of bone marrow mesenchymal stem cells pretreated with valproic acid in rats with an acute spinal cord injury. *Bioscience Trends* 2014;**8**(2): 111–119.

49 Alexanian A, Fehlings MG, Zhang Z, Maiman DJ. Transplanted neurally modified bone marrow-derived mesenchymal stem cells promote tissue protection and locomotor recovery in spinal cord injured rats. *Neurorehabilitation and Neural Repair* 2011;**25**(9): 873–880.

50 Zhang L, Zhang HT, Hong SQ, *et al.* Cografted Wharton's jelly cells-derived neurospheres and BDNF promote functional recovery after rat spinal cord transection. *Neurochemical Research* 2009;**34** (11):2030–2039.

51 Arboleda D, Forostyak S, Jendelova P, *et al.* Transplantation of predifferentiated adipose-derived stromal cells for the treatment of spinal cord injury. *Cellular and Molecular Neurobiology* 2011;**31** (7):1113–1122.

52 Zhang HT, Luo J, Sui LS, *et al.* Effect of differentiated versus undifferentiated adipose tissue-derived stromal cell grafts on functional recovery after spinal cord contusion. *Cellular and Molecular Neurobiology* 2009;**29**(8):1283–1292.

53 Oh JS, Ha Y, An SS, *et al.* Hypoxia-preconditioned adipose tissue-derived mesenchymal stem cell increase the survival and gene expression of engineered neural stem cells in a spinal cord injury model. *Neuroscience Letters* 2010;**472**(3):215–219.

54 Ban DX, Ning GZ, Feng SQ, *et al.* Combination of activated Schwann cells with bone mesenchymal stem cells: the best cell strategy for repair after spinal cord injury in rats. *Regenerative Medicine* 2011;**6**(6):707–720.

55 Bi XB, Deng YB, Gan DH, Wang YZ. Salvianolic acid B promotes survival of transplanted mesenchymal stem cells in spinal cord-injured rats. *Acta Pharmacologica Sinica* 2008;**29**(2):169–176.

56 Torres-Espin A, Redondo Castro E, Hernandez J, Navarro X. Immunosuppression of allogeneic mesenchymal stem cells transplantation after spinal cord injury improves graft survival and beneficial outcomes. *Journal of Neurotrauma* 2015;**32**:367–380.

57 Fang KM, Chen JK, Hung SC, *et al.* Effects of combinatorial treatment with pituitary adenylate cyclase activating peptide and human mesenchymal stem cells on spinal cord tissue repair. *PLoS ONE* 2010;**5**(12):e15299.

58 Shin DA, Pennant WA, Yoon DH, *et al.* Co-transplantation of bone marrow-derived mesenchymal stem cells and nanospheres containing FGF-2 improve cell survival and neurological function in the injured rat spinal cord. *Acta Neurochirurgica* 2014;**156**(2): 297–303.

59 Shang AJ, Hong SQ, Xu Q, *et al.* NT-3-secreting human umbilical cord mesenchymal stromal cell transplantation for the treatment of acute spinal cord injury in rats. *Brain Research* 2011;**1391**:102–113.

60 Chen YF, Zeng X, Zhang K, *et al.* Neurotrophin-3 stimulates migration of mesenchymal stem cells overexpressing TrkC. *Current Medicinal Chemistry* 2013;**20**(24):3022–3033.

61 Ding Y, Yan Q, Ruan JW, *et al.* Electroacupuncture promotes the differentiation of transplanted bone marrow mesenchymal stem cells overexpressing TrkC into neuron-like cells in transected spinal cord of rats. *Cell Transplantation* 2013;**22**(1):65–86.

62 Zhao T, Yan W, Xu K, *et al.* Combined treatment with platelet-rich plasma and brain-derived neurotrophic factor-overexpressing bone marrow stromal cells supports axonal remyelination in a rat spinal cord hemi-section model. *Cytotherapy* 2013;**15**(7): 792–804.

63 Liu WG, Wang ZY, Huang ZS. Bone marrow-derived mesenchymal stem cells expressing the bFGF transgene promote axon regeneration and functional recovery after spinal cord injury in rats. *Neurological Research* 2011;**33**(7):686–693.

64 Jeong SR, Kwon MJ, Lee HG, *et al.* Hepatocyte growth factor reduces astrocytic scar formation and promotes axonal growth beyond glial scars after spinal cord injury. *Experimental Neurology* 2012;**233**(1):312–322.

65 Wu W, Zhao H, Xie B, *et al.* Implanted spike wave electric stimulation promotes survival of the bone marrow mesenchymal stem cells and functional recovery in the spinal cord injured rats. *Neuroscience Letters* 2011;**491**(1):73–78.

66 Ding Y, Yan Q, Ruan JW, *et al.* Electro-acupuncture promotes survival, differentiation of the bone marrow mesenchymal stem cells as well as functional recovery in the spinal cord-transected rats. *BMC Neuroscience* 2009;**10**:35.

67 Yan Q, Ruan JW, Ding Y, *et al.* Electro-acupuncture promotes differentiation of mesenchymal stem cells, regeneration of nerve fibers and partial functional recovery after spinal cord injury. *Experimental and Toxicologic Pathology* 2011;**63**(1–2):151–156.

68 Cho H, Choi YK, Lee DH, *et al.* Effects of magnetic nanoparticle-incorporated human bone marrow-derived mesenchymal stem cells exposed to pulsed electromagnetic fields on injured rat spinal cord. *Biotechnology and Applied Biochemistry* 2013;**60**(6): 596–602.

69 Vanecek V, Zablotskii V, Forostyak S, *et al.* Highly efficient magnetic targeting of mesenchymal stem cells in spinal cord injury. *International Journal of Nanomedicine* 2012;**7**:3719–3730.

70 Jendelova P, Herynek V, Urdzikova L, *et al.* Magnetic resonance tracking of transplanted bone marrow and embryonic stem cells labeled by iron oxide nanoparticles in rat brain and spinal cord. *Journal of Neuroscience Research* 2004;**76**(2):232–243.

71 Chekina N, Horak D, Jendelova P, *et al.* Fluorescent magnetic nanoparticles for biomedical applications. *Journal of Materials Chemistry* 2011;**21**(21):7630–7639.

72 Hejcl A, Lesny P, Pradny M, *et al.* Biocompatible hydrogels in spinal cord injury repair. *Physiological Research/Academia Scientiarum Bohemoslovaca* 2008;**57**(Suppl 3):S121–S132.

73 Kubinova S, Sykova E. Biomaterials combined with cell therapy for treatment of spinal cord injury. *Regenerative Medicine* 2012;**7** (2):207–224.

74 Hejcl A, Urdzikova L, Sedy J, *et al.* Acute and delayed implantation of positively charged 2-hydroxyethyl methacrylate scaffolds in

spinal cord injury in the rat. *Journal of Neurosurgery Spine* 2008;**8**(1):67–73.
75 Chen J, Zhang Z, Liu J, *et al.* Acellular spinal cord scaffold seeded with bone marrow stromal cells protects tissue and promotes functional recovery in spinal cord-injured rats. *Journal of Neuroscience Research* 2014;**92**(3):307–317.
76 Liu J, Chen J, Liu B, *et al.* Acellular spinal cord scaffold seeded with mesenchymal stem cells promotes long-distance axon regeneration and functional recovery in spinal cord injured rats. *Journal of the Neurological Sciences* 2013;**325**(1–2):127–136.
77 Hejcl A, Sedy J, Kapcalova M, *et al.* HPMA-RGD hydrogels seeded with mesenchymal stem cells improve functional outcome in chronic spinal cord injury. *Stem Cells and Development* 2010;**19**(10):1535–1546.
78 Kang KN, Kim da Y, Yoon SM, *et al.* Tissue engineered regeneration of completely transected spinal cord using human mesenchymal stem cells. *Biomaterials* 2012;**33**(19):4828–4835.
79 Hodgetts SI, Simmons PJ, Plant GW. A comparison of the behavioral and anatomical outcomes in sub-acute and chronic spinal cord injury models following treatment with human mesenchymal precursor cell transplantation and recombinant decorin. *Experimental Neurology* 2013;**248**:343–359.
80 Zurita M, Vaquero J, Bonilla C, *et al.* Functional recovery of chronic paraplegic pigs after autologous transplantation of bone marrow stromal cells. *Transplantation* 2008;**86**(6):845–853.
81 Lu P, Jones LL, Tuszynski MH. Axon regeneration through scars and into sites of chronic spinal cord injury. *Experimental Neurology* 2007;**203**(1):8–21.
82 Knutsdottir S, Thorisdottir H, Sigvaldason K, *et al.* Epidemiology of traumatic spinal cord injuries in Iceland from 1975 to 2009. *Spinal Cord* 2012;**50**(2):123–126.
83 Sekhon LH, Fehlings MG. Epidemiology, demographics, and pathophysiology of acute spinal cord injury. *Spine* 2001;**26**(24 Suppl):S2–S12.
84 Wyndaele JJ. Outcome after spinal cord lesion: relation to country, social support, education, income and ways to measure outcome. *Spinal Cord* 2009;**47**(8):577–578.
85 Wyndaele JJ. More knowledge of worldwide incidence and epidemiology of spinal cord injury: data from the United States military. *Spinal Cord* 2011;**49**(8):857.
86 Wyndaele M, Wyndaele JJ. Incidence, prevalence and epidemiology of spinal cord injury: what learns a worldwide literature survey? *Spinal Cord* 2006;**44**(9):523–529.
87 Hughes JT. The Edwin Smith Surgical Papyrus: an analysis of the first case reports of spinal cord injuries. *Paraplegia* 1988;**26**(2):71–82.
88 Fehlings MG, Vaccaro A, Wilson JR, *et al.* Early versus delayed decompression for traumatic cervical spinal cord injury: results of the Surgical Timing in Acute Spinal Cord Injury Study (STASCIS). *PLoS ONE* 2012;**7**(2):e32037.
89 Bracken MB. Methylprednisolone and acute spinal cord injury: an update of the randomized evidence. *Spine* 2001;**26**(24 Suppl):S47–S54.
90 Harrop JS. Spinal cord injury: debating the efficacy of methylprednisolone. *Neurosurgery* 2014;**61**(Suppl 1):30–31.
91 Syková E, Homola A, Mazanec R, *et al.* Autologous bone marrow transplantation in patients with subacute and chronic spinal cord injury. *Cellular Transplantation* 2006;**15**(8–9):675–687.
92 Park HC, Shim YS, Ha Y, *et al.* Treatment of complete spinal cord injury patients by autologous bone marrow cell transplantation and administration of granulocyte-macrophage colony stimulating factor. *Tissue Engineering* 2005;**11**(5–6):913–922.
93 Yoon SH, Shim YS, Park YH, *et al.* Complete spinal cord injury treatment using autologous bone marrow cell transplantation and bone marrow stimulation with granulocyte macrophage-colony stimulating factor: phase I/II clinical trial. *Stem Cells* 2007;**25**(8):2066–2073.
94 Callera F, do Nascimento RX. Delivery of autologous bone marrow precursor cells into the spinal cord via lumbar puncture technique in patients with spinal cord injury: a preliminary safety study. *Experimental Hematology* 2006;**34**(2):130–131.
95 Kumar AA, Kumar SR, Narayanan R, *et al.* Autologous bone marrow derived mononuclear cell therapy for spinal cord injury: a phase I/II clinical safety and primary efficacy data. *Experimental and Clinical Transplantation* 2009;**7**(4):241–248.
96 Deda H, Inci MC, Kurekci AE, *et al.* Treatment of chronic spinal cord injured patients with autologous bone marrow-derived hematopoietic stem cell transplantation: 1-year follow-up. *Cytotherapy* 2008;**10**(6):565–574.
97 Geffner LF, Santacruz P, Izurieta M, *et al.* Administration of autologous bone marrow stem cells into spinal cord injury patients via multiple routes is safe and improves their quality of life: comprehensive case studies. *Cell Transplantation* 2008;**17**(12):1277–1293.
98 Kishk NA, Gabr H, Hamdy S, *et al.* Case control series of intrathecal autologous bone marrow mesenchymal stem cell therapy for chronic spinal cord injury. *Neurorehabilitation and Neural Repair* 2010;**24**(8):702–708.
99 Dai G, Liu X, Zhang Z, *et al.* Transplantation of autologous bone marrow mesenchymal stem cells in the treatment of complete and chronic cervical spinal cord injury. *Brain Research* 2013;**1533**:73–79.
100 Karamouzian S, Nematollahi-Mahani SN, Nakhaee N, Eskandary H. Clinical safety and primary efficacy of bone marrow mesenchymal cell transplantation in subacute spinal cord injured patients. *Clinical Neurology and Neurosurgery* 2012;**114**(7):935–939.
101 Pal R, Venkataramana NK, Bansal A, *et al. Ex vivo*-expanded autologous bone marrow-derived mesenchymal stromal cells in human spinal cord injury/paraplegia: a pilot clinical study. *Cytotherapy* 2009;**11**(7):897–911.
102 Bhanot Y, Rao S, Ghosh D, *et al.* Autologous mesenchymal stem cells in chronic spinal cord injury. *British Journal of Neurosurgery* 2011;**25**(4):516–522.
103 Ra JC, Shin IS, Kim SH, *et al.* Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells and Development* 2011;**20**(8):1297–1308.
104 Cheng H, Liu X, Hua R, *et al.* Clinical observation of umbilical cord mesenchymal stem cell transplantation in treatment for sequelae of thoracolumbar spinal cord injury. *Journal of Translational Medicine* 2014;**12**(1):253.
105 Liu J, Han D, Wang Z, *et al.* Clinical analysis of the treatment of spinal cord injury with umbilical cord mesenchymal stem cells. *Cytotherapy* 2013;**15**(2):185–191.

106 Hammadi AA, Marino A, Farhan S. Clinical response of 277 patients with spinal cord injury to stem cell therapy in Iraq. *International Journal of Stem Cells* 2012;**5**(1):76–78.

107 Saito F, Nakatani T, Iwase M, *et al.* Administration of cultured autologous bone marrow stromal cells into cerebrospinal fluid in spinal injury patients: a pilot study. *Restorative Neurology and Neuroscience* 2012;**30**(2):127–136.

108 Park JH, Kim DY, Sung IY, *et al.* Long-term results of spinal cord injury therapy using mesenchymal stem cells derived from bone marrow in humans. *Neurosurgery* 2012;**70**(5):1238–1247, discussion 1247.

109 Jiang PC, Xiong WP, Wang G, *et al.* A clinical trial report of autologous bone marrow-derived mesenchymal stem cell transplantation in patients with spinal cord injury. *Experimental and Therapeutic Medicine* 2013;**6**(1):140–146.

110 Amr SM, Gouda A, Koptan WT, *et al.* Bridging defects in chronic spinal cord injury using peripheral nerve grafts combined with a chitosan–laminin scaffold and enhancing regeneration through them by co-transplantation with bone-marrow-derived mesenchymal stem cells: case series of 14 patients. *The Journal of Spinal Cord Medicine* 2014;**37**(1):54–71.

111 Moviglia GA, Varela G, Brizuela JA, *et al.* Case report on the clinical results of a combined cellular therapy for chronic spinal cord injured patients. *Spinal Cord* 2009;**47**(6):499–503.

112 Cristante AF, Barros-Filho TE, Tatsui N, *et al.* Stem cells in the treatment of chronic spinal cord injury: evaluation of somatosensitive evoked potentials in 39 patients. *Spinal Cord* 2009;**47**(10):733–738.

CHAPTER 50

# The role of mesenchymal stromal cells in the treatment of ulcerative colitis and Crohn's disease

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## 50.1 Inflammatory bowel diseases

Inflammatory bowel diseases (IBDs) are chronic relapsing diseases associated with debilitating symptoms and leading to progressive intestinal tissue damage and disability (Figure 50.1).

Crohn's disease (CD) can affect any part of the gastrointestinal tract, and lesions are often segmental and transmural. The disease was named after gastroenterologist Dr Burrill Bernard Crohn, who, in 1932, together with two other colleagues at Mount Sinai Hospital in New York, described a series of patients with inflammation of the terminal ileum of the small intestine, the area most commonly affected by the illness. Active CD is characterized by focal inflammation with granulomas and formation of fistula tracts or abscesses, and chronic lesions include fibrosis and stricture of the bowel (Figure 50.2).

In ulcerative colitis (UC), mucosal inflammation is continuous and limited to the mucosa and superficial submucosa. Lesions can affect the rectum and to a variable extent the colon, without granulomas or fistulas. The age of onset of IBD is most frequently in late adolescence or early adulthood, and the disease is equally

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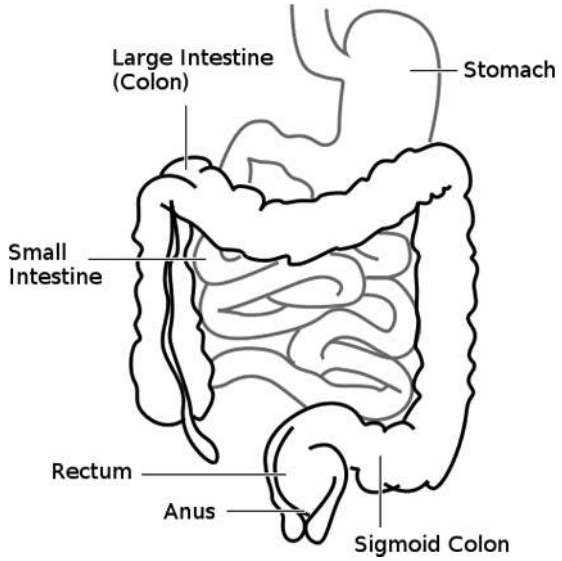



**Figure 50.1** Diagram of the human intestine. Drawn by Duncan Lock and released into the Public Domain. *Source:* http://commons.wikimedia.org/wiki/File:Intestine-diagram.svg#mediaviewer/File:Intestine-diagram.svg.

distributed between the sexes. The incidence and prevalence are stable in high-incidence areas (northern Europe and North America), and rising in low-incidence areas (southern Europe, Asia, and most developing countries) [1,2].

Symptoms of CD are heterogeneous, but commonly include chronic diarrhea, abdominal pain, weight loss, anorexia, and/or fever. Rectal bleeding occurs commonly in UC, while perianal fissures, fistulas, or abscesses can occur in CD. Extraintestinal features can be related to extraintestinal inflammation, such as joint inflammation (spondyloarthritis, peripheral arthritis),



**Figure 50.2** A portion of resected jejunum showing the typical cobblestone appearance of CD affecting the jejunum. *Source:* http://commons.wikimedia.org/wiki/File:Crohn_Jejunum.PNG#mediaviewer/File:Crohn_Jejunum.PNG.



**Figure 50.3** Erythema nodosum is an inflammatory condition characterized by inflammation of the fat cells under the skin, resulting in tender red nodules or lumps. It can be caused by a variety of conditions and typically resolves spontaneously within 3–6 weeks. The commonest distribution of erythema nodosum is on the shins – the anterior aspect of the lower legs. *Source:* 1905, William Wood and Company.

cutaneous manifestations – erythema nodosum (Figure 50.3), pyoderma gangrenosum (Figure 50.4) – ocular inflammation (uveitis, episcleritis, sclero-conjunctivitis), and primary sclerosing cholangitis, or are consequences of malabsorption (cholelithiasis, nephrolithiasis, and osteoporosis) [3].

The diagnosis is based on clinical evaluation and a combination of endoscopic, histological, radiological (computed tomography scan, magnetic resonance imaging (MRI)), and/or biochemical (C-reactive protein (CRP), full blood count, and fecal calprotectin) investigations. The histologic diagnosis is based on the presence of discontinuous chronic inflammation, focal crypt irregularity, and granulomas in CD [4].

## 50.2 Pathogenesis of inflammatory bowel diseases

The etiology of IBD is multifactorial, polygenic [5], and environmental [6] and includes an abnormal systemic and mucosal




**Figure 50.4** Pyoderma gangrenosum affecting the lower leg of a person with CD. This is a condition that causes tissue to become necrotic, resulting in deep ulcers that usually occur on the legs. Ulcers usually initially look like small bug bites or papules and then progress to larger ulcers. While the ulcers rarely lead to death, they can cause pain and scarring. Although the etiology is not well understood, the disease is thought to be due to immune system dysfunction, and particularly abnormal functioning of neutrophils. The commonest diseases associated with pyoderma gangrenosum are the IBDs (CD and UC), followed by the arthritides such as rheumatoid arthritis, followed by malignant hematological disorders. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Crohnie_Pyoderma_gangrenosum.jpg#file.

immune response against intraluminal antigens, favored by microbial factors [7] and alteration of the mucosal barrier [8]. A key role in the pathogenesis of IBD seems to be played by enhanced proliferation and defective apoptosis of immune cells, attributed to an imbalance of the antiapoptotic protein BCL-2 and the pro-apoptotic protein BAX [9,10]), as well as by proinflammatory cytokines arising from T helper cells (Th)1 and Th17 CD4 T cell differentiation in CD, and Th2 T cell differentiation in UC [11–13]. The loss of balance between proliferation and apoptosis is responsible for the presence of an abnormal population of T cells with extended lifespan, which remains in the mucosal compartment and secretes proinflammatory cytokines. The result is an activation of other inflammatory cells, including macrophages and B cells, and a recruitment of circulating leukocytes into the gut through interactions between homing receptors (e.g., $\alpha_4\beta_7$ integrin) and addressins on vascular endothelium, such as the mucosal vascular addressin cell adhesion molecule (MAdCAM)-1 or the vascular cell adhesion molecule (VCAM)-1.

In CD the initial phase of the inflammation seems to be predominantly driven by a Th1 response. Interleukin (IL)-12, a proinflammatory cytokine produced by dendritic cells (DCs), promotes the conversion of T cells into Th1 cells, secreting interferon (INF)-γ and tumor necrosis factor (TNF)-α, two major actors in the induction and propagation of inflammation [14–18]. In a later phase, the importance of the Th17 response increases. Th17 cells are induced by IL-6 and transforming growth factor (TGF)-β, and secrete IL-17 and IL-22. IL-23, produced by DCs, interacts with Th17 cells and increases the secretion of IL-6 and IL-17 by T cells, thus enhancing the inflammatory response [19–24]. This overactive immune response can lead to the development of mucosal lesions. Defective apoptosis also promotes autoimmunity, by a reduced autoantibody clearance. Interestingly, almost all drugs with clinical efficacy, including infliximab [25], are able to induce apoptosis of immune cells *in vitro*, while etanercept, an anti-TNF-fusion protein that does not induce apoptosis of immune cells [26], is not efficacious in the treatment of CD [27]. Nevertheless, certolizumab pegol, another anti-TNF agent, is unable to induce cell apoptosis [28], and yet is effective for inducing and maintaining remission in active CD [29,30]. Eventually, chronic inflammation induces intestinal fibroblasts to proliferate and produce a greater amount of extracellular matrix, resulting in local intestinal fibrosis [31].

Endoscopic appearances are shown in Figure 50.5.

In UC, inflammation is mainly driven by IL-5 and IL-13, while production of INF-γ is normal. The disease is qualified as "Th2-like" given the normal production of IL-4, the most defining Th2 cytokine [11]. A Th17 response may also play a role, but its importance is probably lower than in CD.

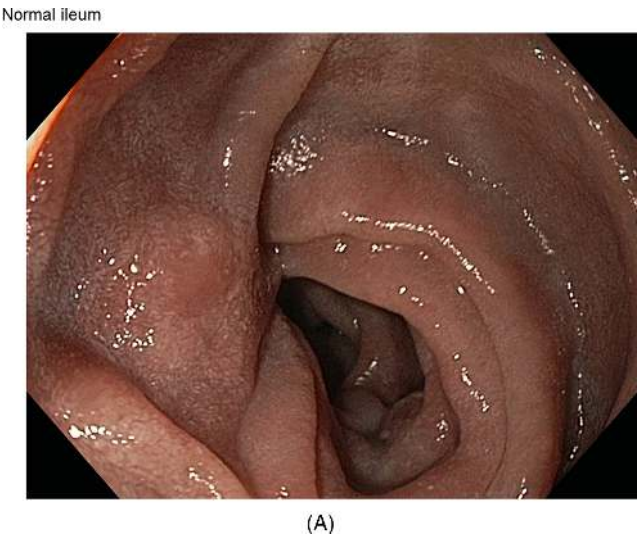



**Figure 50.5** (A) Normal ileum: the mucosa is homogeneously pink and the villi are regular and well developed. (B) Crohn's ileitis: diffuse inflammation characterized by erythema and mucosal hemorrhage. (C) Crohn's ileitis: presence of erythema and ulcers; red dots indicate mucosal hemorrhages. (D) Normal colon: colonic folds are present and regular. The vascular pattern is also readily visible. (E, F) Crohn's colitis: presence of diffuse inflammation with erythema and superficial ulcers. (G) Healed colon. (See color plate section.)

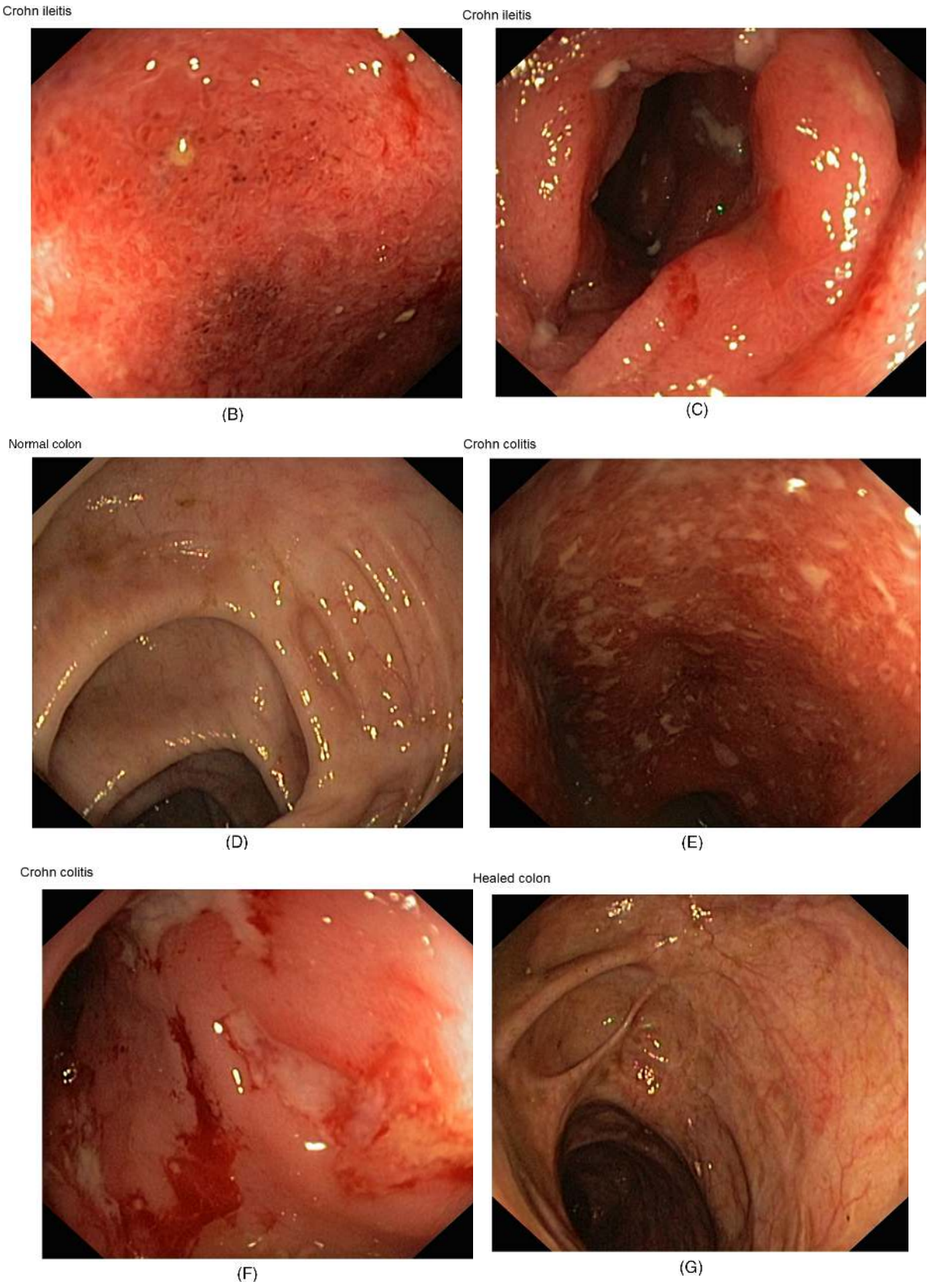



**Figure 50.5** (*Continued*)

**Table 50.1** The CDAI is a research tool used to quantify the symptoms of patients with CD.

| Clinical or laboratory variable | Weighting factor |
|---|---|
| Number of liquid or soft stools each day for 7 days | ×2 |
| Abdominal pain (graded from 0 to 3 on severity) each day for 7 days | ×5 |
| General wellbeing, subjectively assessed from 0 (well) to 4 (terrible) each day for 7 days | ×7 |
| Presence of complications[a] | ×20 |
| Taking Lomotil or opiates for diarrhea | ×30 |
| Presence of an abdominal mass (0 as none, 2 as questionable, 5 as definite) | ×10 |
| Hematocrit of <0.47 in men and <0.42 in women | ×6 |
| Percentage deviation from standard weight | ×1 |

Source: Best et al. [32].
Remission of CD is defined as CDAI below 150. Severe disease is defined as a value of greater than 450.
[a] One point each is added for each set of complications: (a) the presence of joint pains (arthralgia) or frank arthritis; (b) inflammation of the iris or uveitis; (c) the presence of erythema nodosum, pyoderma gangrenosum, or aphthous ulcers; (d) the presence anal fissures, fistulae, or abscesses; (e) the presence of other fistulae; (f). Fever during the previous week.

## 50.3 Treatment of Crohn's disease

The choice of therapy is based on anatomic location, severity, and behavior of disease. Severity can be assessed by the CD activity index (CDAI), a score based on daily symptoms and repercussions of CD such as hematocrit, weight, and other factors (Table 50.1).

CD can be classified as mild or mild–moderate (CDAI between 150 and 220), moderate or moderate–severe (CDAI between 220 and 450), or severe or severe/fulminant (CDAI >450). There is a current trend to use CRP levels in conjunction with the CDAI to evaluate severity of the disease [4,33]. Disease remission is defined in the majority of clinical trials as a CDAI of less than 150 [34]. Response is often defined by a variation of CDAI of 100 points or more, although a variation of 70 points or more has been used in some studies. The perineal disease activity index (PDAI) is sometimes used if perineal lesions are predominant. Endoscopic evaluation can be standardized using the CD endoscopic index of severity (CDEIS) or a simplified variant, the simple endoscopic score for CD (SESCD) [35].

### 50.3.1 Current treatment options for Crohn's disease

Current treatment strategies include oral 5-aminosalicylates (sulfasalazine, mesalamine), antibiotics (metronidazole, ciprofloxacin), topically or systemically acting steroids (controlled ileal release budesonide or prednisone), and immunomodulator therapy, including azothioprine, 6-mercaptopurine, methotrexate, calcineurin inhibitors (cyclosporine, tacrolimus) and anti-TNF antibodies (infliximab, adalimumab, certolizumab pegol). Nutritional therapy (elemental or polymeric diets) can be considered as an adjunct treatment. Mildly to moderately active localized ileocecal disease can often be managed with budesonide, while severe active or extensive disease generally requires systemic corticosteroids with or without an immunomodulator. Anti-TNF therapy, used alone or in adjunction to an immunomodulator, is recommended in moderate to severe or extensive relapsing disease or in previously steroid-refractory, -dependent, or -intolerant patients. All currently available anti-TNF therapies appear to have similar efficacy.

If there is loss of response to anti-TNF therapy, increasing doses or frequency of administration can be tried before switching to another anti-TNF agent. Surgery should be considered in localized disease. Treatment of relapse is essentially based upon previously successful therapies, but other factors such as patient preference, time to relapse, concurrent therapy, and adherence to therapy are taken into account. The choice of maintenance therapy is essentially based upon the course and extent of disease, and the effectiveness and tolerance of previously used treatments. Some patients do not need maintenance. Azathioprine is generally the first choice, followed by methotrexate and anti-TNF therapy [36].

Perianal and fistulizing disease can be treated by surgery, including surgical drainage, fistulotomy and setons, advancement flaps, antibiotics, or an immunomodulator (including anti-TNF agents) [33]. A seton or seton stitch is a procedure used to aid the healing of fistulas. The procedure involves running a surgical-grade cord through the fistula tract so that the cord creates a loop that joins up outside the fistula. The cord provides a path that allows the fistula to drain continuously while it is healing, rather than allowing the exterior of the wound to close over. Keeping the fistula tract open can help keep from trapping pus or other infectious material in the wound. The procedure was mentioned by Hippocrates in 400 BC. Flap surgery is a technique in plastic and reconstructive surgery where any type of tissue is lifted from a donor site and moved to a recipient site with an intact blood supply. Advancement flaps are used when the patient is in overall good health and the defect is free of tumor and obvious infection.

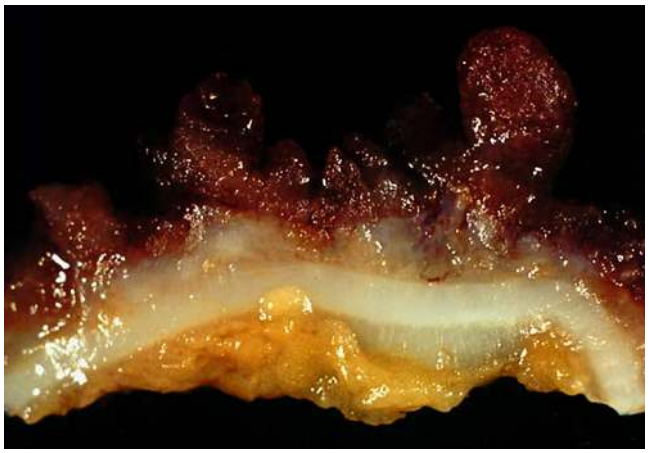


**Figure 50.6** Chronic UC. The image shows a view of a longitudinal section through the colon wall. This demonstrates not only the angry red mucosa but also the tendency for the inflamed tissue to throw itself up into inflammatory pseudopolyps. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Chronic_Ulcerative_Colitis_1.jpg#mediaviewer/File:Chronic_Ulcerative_Colitis_1.jpg.

Whereas 80–90% of the patients can be satisfactorily treated with these approaches, some patients are refractory, intolerant, or lose response to them. In this setting, few options are left apart from debilitating surgery with a permanent stoma or experimental treatments. Images of surgical resections are shown in Figures 50.6 and 50.7.

### 50.3.2 Potential treatment options

New therapies that have demonstrated some efficacy are inhibiting leucocyte migration, targeting inflammatory cytokines, enhancing immune regulatory mechanisms, and/or promoting tissue repair.



**Figure 50.7** Colonic pseudopolyps of a patient with chronic severe intractable UC. Colectomy specimen. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Chronic_Ulcerative_Colitis_1.jpg#mediaviewer/File:Chronic_Ulcerative_Colitis_1.jpg.

#### 50.3.2.1 Drugs targeting leucocyte homing

Alpha-4 integrins are members of the cell-surface adhesion family expressed by several cells, including leucocytes, and binding several ligands, including VCAM-1 expressed on endothelial cells, and extra-cellular matrix proteins such as fibronectin. These interactions lead to various cellular processes, such as activation, proliferation, and migration of $\alpha_4$-integrin-expressing cells toward sites of inflammation, including areas of the gut in CD [37]. Natalizumab, a humanized monoclonal antibody against the $\alpha_4$ subunit of both $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins, has demonstrated therapeutic benefit in patients with moderate to severe active CD. In the ENACT-1 randomized clinical trial (RCT) evaluating natalizumab versus placebo as induction therapy in 905 patients with active CD, similar rates of response (decrease in CDAI of ≥70 points) and remission (CDAI <150) were observed at week 10 (respectively 56% versus 49%, $P = 0.05$, and 37% versus 30%, $P = 0.12$). In a second part of the trial (ENACT-2) evaluating natalizumab versus placebo as maintenance therapy in 339 patients who had a response to natalizumab in the first trial, a significantly higher rate of sustained response (61% versus 28%, $P < 0.001$) and remission (44% versus 26%, $P = 0.003$) was observed at week 36 [38]. In ENCORE, an RCT evaluating the efficacy of natalizumab versus placebo as induction therapy in 509 patients with moderately to severely active CD and active inflammation (CRP >2.87 mg/L), significantly higher rates of response (decrease in CDAI of ≥70 points) at week 8 and remission (CDAI <150) were observed (respectively 48% versus 32%, $P < 0.001$, and 26% versus 16%, $P = 0.002$) [39]. In a meta-analysis of five trials comparing natalizumab with placebo, the relative risk (RR) of not achieving remission was reduced with natalizumab (0.88) [40]. In the USA, monotherapy with natalizumab is indicated for inducing and maintaining clinical response and remission in adult patients with moderately to severely active CD and evidence of inflammation who have had an inadequate response or who are unable to tolerate conventional CD therapies and anti-TNF agents [33]. The most dangerous adverse effect is progressive multifocal leukoencephalopathy caused by the JC virus.

Vedolizumab, a humanized anti-$\alpha_4\beta_7$ integrin monoclonal antibody, has also been approved by the US Food and Drug Administration and the European Medicine Agency for use in patients with moderate to severe CD. In the induction phase comparing intravenous vedolizumab therapy (300 mg) versus placebo in 368 adults with active CD, a higher rate of clinical remission (CDAI ≤150) was observed (14.5% versus 6.8%, $P = 0.02$), while CDAI-100 response was similar (31.4% versus 25.7%, $P = 0.23$). In the maintenance phase comparing intravenous vedolizumab therapy every 8 or 4 weeks versus placebo in 461 patients who had had a response to vedolizumab, there was a significantly higher rate of clinical remission (39.0% and 36.4% for the two vedolizumab groups, respectively, versus 21.6% for the placebo group; $P < 0.001$ and $P = 0.004$), but also a higher rate of serious adverse events (24.4% versus 15.3%) and infections (44.1% versus 40.2%) [41].

Chemokine receptor 9 (CCR9) is a highly specific receptor expressed predominately by T cells. Its overexpression leads to the migration of T cells to the small and large intestines. In PROTECT-1, an RCT with 436 subjects suffering from moderate to severe CD (CDAI between 250 and 450 and CRP >7.5 mg/L), a CCR9 antagonist (CCX282-b/Traficet-EN) given 250 mg twice daily for 36 weeks demonstrated efficacy in maintaining remission in patients identified as responders (decrease in CDAI of ≥70 points) after the induction phase (47% of subjects on CCX282-B in remission at 36 weeks versus 31% on placebo; $P = 0.01$). CCX282-B was also superior to placebo in normalizing CRP at week 36 (19% versus 9%; $P = 0.04$), and decreasing the number of subjects requiring corticosteroid rescue therapy (11% versus 21%, $P = 0.04$). Serious adverse events occurred in similar rates in the two groups [42].

Other molecules targeting T cells, but not interfering with homing, have been studied with less success. Visilizumab, a humanized monoclonal antibody directed at the cell surface marker CD3 expressed only by activated T cells, inducing selective apoptosis of these activated T cells, has shown some efficacy in an uncontrolled trial [43] but was associated with an increased rate of adverse events, including cytokine release syndrome and liver toxicity [44].

Cytokine release syndrome is a common immediate complication occurring with the use of anti-T cell antibody infusions such as antithymocyte globulin (ATG), OKT3 (muromonab-CD3), and TGN1412, and also with the CD-20 antibody rituximab against B cells. Severe cases are known as cytokine storms. The antibodies bind to the T cell receptor (TCR), activating the T cells before they are destroyed. The cytokines released by the activated T cells produce a type of systemic inflammatory response similar to that found in severe infections and characterized by hypotension, pyrexia, and rigors. The patient feels very unwell, as if in a high fever – indeed, the cytokine release syndrome *is* effectively a type of noninfective fever. Deaths due to cytokine release syndrome with OKT3 have been reported, and it can cause life-threatening pulmonary edema if the patient is fluid overloaded. However, if treated appropriately it is usually not dangerous, just extremely unpleasant for the patient.

Abatacept, a CTLA-Ig (cytotoxic T-lymphocyte-associated protein) that blocks the co-activation of T cells by competing with CD28 for CD80/CD86, and which is used in the treatment of rheumatoid arthritis, was not found to be effective in CD [45].

#### 50.3.2.2 Drugs targeting the proinflammatory cascade

The proinflammatory cytokines IL-12 and IL-23 are involved in Th1 and Th17 responses respectively, and both are implicated in the pathogenesis of CD [46]. Ustekinumab, a human monoclonal antibody blocking the p40 common subunit of IL-12 and IL-23, was evaluated in a phase II RCT with 526 adults suffering from moderate-to-severe CD that was resistant to TNF antagonists. In the induction phase, clinical response at 6 weeks was significantly higher in a subgroup of patients receiving 6 mg/kg of ustekinumab intravenously (39.7% versus 23.5% in the placebo group, $P = 0.005$), while there was no significant difference in clinical remission. In the maintenance phase of the trial, comparing 90 mg of ustekinumab subcutaneously versus placebo in 145 patients who had a response to ustekinumab at 6 weeks, significantly increased rates of clinical response (69.4% versus 42.5%, $P < 0.001$) and remission (41.7% versus 27.4%, $P = 0.03$) at 22 weeks [47]. Phase III trials are currently underway to evaluate the safety and efficacy of ustekinumab (www.clinicaltrials.gov; NCT01369355, NCT01369342, NCT01369329).

The proinflammatory cytokine IL-6 has been implicated in the pathogenesis of colitis in various animal models and in patients with CD [48,49]. In a small pilot study with 36 patients suffering from active CD, a biweekly 8 mg/kg infusion of tocilizumab, an anti-IL-6 receptor humanized monoclonal antibody used in rheumatoid arthritis, showed promising results with 8/10 (80%) patients experiencing a clinical response compared with 4/13 (31%) patients in the placebo group ($P = 0.019$), although only two patients achieved remission. The incidence of adverse events was similar in the two groups [50].

Thalidomide has anti-inflammatory effects, by decreasing the production of proinflammatory cytokines, including TNF-α and IL-12. It appears to be effective in inducing remission in refractory pediatric CD, as shown in a randomized controlled trial with 56 children with active CD despite immunosuppressive treatment (prolonged by an open-label extension of the study, where nonresponders to placebo received thalidomide). Overall, 32/49 (65.3%) patients treated with thalidomide achieved a response (reduction in pediatric CDAI by ≥75%) and 31/49 (63.3%) achieved clinical remission [51].

Since IL-17 and IFN-γ have been identified as major actors in the pathogenesis of CD, molecules inhibiting these agents have been developed. However, anti-IL-17 antibodies (secukinumab [52], brodalumab [53]) and anti-IFN-γ antibodies (fontolizumab [54]) all failed to demonstrate efficacy. Despite encouraging preliminary results, attempts to administer anti-inflammatory cytokines, such as IL-10 [55,56] and IL-11 [57,58], were also disappointing, as no significant improvement was observed.

#### 50.3.2.3 Other treatments

Some other treatments have been studied with encouraging preliminary results, including human growth hormone [59], linoleic acid [60], low-dose naltrexone [61], helminths [62,63], or extracorporeal photopheresis [64]. Larger studies are needed to confirm these results. Other agents have so far failed to demonstrate efficacy including granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) [65,66], probiotics [67–70], and prebiotics [71]. If intestinal failure occurs, home parenteral nutrition and intestinal transplantation can be discussed [72]. Interestingly, several cases of CD recurrence after intestinal transplantation have been reported [73–75], which may indicate that the immune system plays a more important role than the mucosal barrier in the pathogenesis of CD.

#### 50.3.2.4 Bone marrow transplantation and stem cell therapy

The first reported success of autologous hematopoietic stem cell transplantation (HSCT) in CD was published in 1993 in a patient with non-Hodgkin's lymphoma [76]. Since then, at least 19 cases of autologous or allogeneic HSCTs for malignancies in CD patients have been reported, with remission of CD achieved in most cases, even after discontinuation of any immunosuppressive therapy [77]. Given these results, HSCT trials in patients with CD refractory to conventional therapy were initiated. In 1995, the Autoimmune Diseases Working Party of the European Group for Blood and Marrow Transplantation recommended the use of autologous rather than allogeneic HSCT because of the lower risk of severe toxicity [78]. The largest study is a phase I–II trial on 24 patients with severe CD (CDAI >250 and/or Crohn Severity Index >16) despite anti-TNF therapy, who underwent autologous HSCT after mobilization of their peripheral blood hematopoietic stem cells (HSCs) with cyclophosphamide and G-CSF, *ex vivo* HSC enrichment by $CD34^+$ selection, and non-marrow ablative preparation with cyclophosphamide and equine or rabbit ATG.

All patients went into remission (CDAI <150). After 5 years, the percentage of clinical relapse-free survival was 19% and the percentage of patients in remission, steroid-free, or medication-free was at least 70%, 80%, and 60% respectively. Infectious complications were frequent, including early bacteremia and/or central venous line infection (six patients) and late infections (10 cases, including seven severe infections, in the first year after transplantation) [79]. A randomized phase III study was initiated, but recruitment was halted prematurely for safety reasons (http://clinicaltrials.gov/ct2/show/NCT00297193). Preliminary results on 48 patients after 1 year follow-up showed decreases in mean CDAI from 324 to 161, CRP from 16.6 to 6.5 mg/L, with six patients achieving a normal CDAI and three patients allowed to stop immune-suppressive medications. However, high rates of adverse events were observed (100 serious adverse events, among which 42 infective episodes requiring or prolonging hospitalization and eight patients with temporary flare of CD activity or a need for surgery) [80].

## 50.4 Treatment of ulcerative colitis

The choice of therapy is based on the activity, distribution, and pattern of disease. Disease activity can be evaluated by Truelove and Witts' criteria (number of bloody stools/day, pulse, temperature, hemoglobin, and erythrocyte sedimentation rate or CRP) or the Mayo score, and is classified as mild, moderate, or severe. Remission is defined as complete resolution of symptoms and endoscopic mucosal healing. Response is defined as clinical and endoscopic improvement (generally a decrease in the activity index of >30%, associated with a decrease in the rectal bleeding and endoscopic scores in the Mayo score) [2].

### 50.4.1 Current treatment options for ulcerative colitis

Current treatment strategies include oral 5-aminosalicylates (sulfasalazine, mesalamine), antibiotics (metronidazole, ciprofloxacin), topically or systemically acting steroids (prednisone, beclomethasone dipropionate), and immunomodulatory therapy, including azathioprine, 6-mercaptopurine, calcineurin inhibitors (cyclosporine, tacrolimus), and anti-TNF antibodies (infliximab, adalimumab, golimumab). Surgery (procolectomy with ileo-anal anastomosis) is an option to treat acute severe colitis or to manage refractory disease (see Figures 50.5 and 50.7) [81].

### 50.4.2 Potential new treatment options

The most promising therapies for UC are essentially acting through an inhibition of leukocyte migration or inflammatory cytokines. Several anti-integrin antibodies have been studied in UC, including vedolizumab [82], which has been recently approved by the US Food and Drug Administration, and etrolizumab, a humanized monoclonal antibody targeting the $\beta_7$ subunit of $\alpha_4\beta_7$ and $\alpha_E\beta_7$) [83,84] and PF-00547,659, a monoclonal antibody to MAdCAM [85], which have been found effective in preliminary trials.

Alicaforsen, an inhibitor of intracellular adhesion molecule-1 [86–88], and tofactinib, a Janus kinase-3 inhibitor [89], have also shown promising results in UC. The roles of methotrexate [90–94], probiotics, and fecal microbiota transplantation [95,96] in the treatment of UC are still debated, and randomized controlled trials are needed to evaluate their effectiveness. Despite encouraging preliminary results, anti-IL-2 receptor (daclizumab [97,98], basiliximab [99–101]) and anti-CD3 (visilizumab [102–104]) monoclonal antibodies have failed to demonstrate efficacy in larger trials. Rituximab (an anti-CD20 antibody against B cells) is ineffective and can even mediate exacerbation of UC [105–107].

## 50.5 Properties of mesenchymal stromal cells

Mesenchymal stromal cells (MSCs) are multipotent progenitors within the bone marrow and all other vascularized organs that are capable of differentiating into various cells and tissues, such as chondrocytes, osteoblasts, and adipocytes *in vitro* [108]. MSCs can be isolated after *ex vivo* culture of the plastic-adherent mononuclear cell fraction. After *ex vivo* expansion human MSCs have a fibroblast-like morphology, and are uniformly positive for CD29, CD44, CD71, CD73, CD90, CD105, CD106, CD120a, CD124, and CD166 but do not express common hematopoietic markers such as CD14, CD45, or CD34. No marker specific for MSCs has been found yet. The International Society for Cellular Therapy has proposed three minimal criteria to define MSCs: (1) adhesion to plastic in standard culture conditions, (2) expression of CD105, CD73, and CD90, and lack of expression of CD45, CD34, CD14 or CD11b, CD79a or CD19, and human leukocyte antigen (HLA)-DR surface

molecules, and (3) *in vitro* differentiation into osteoblasts, adipocytes, and chondroblasts [109]. MSCs can be isolated from bone marrow and many other tissues, including umbilical cord [110], umbilical cord blood [111], placenta [112], adipose tissue [113], gingival tissue [114], skin [115], lung [116], liver [117], heart, and spleen [118]. They are pericytes residing in the endothelium in the connective tissues of most organs [119].

### 50.5.1 Immunomodulation

MSCs also exert powerful immunomodulatory effects, including inhibition of proliferation and function of T cells, B cells, and natural killer (NK) cells. These properties have been demonstrated *in vitro* with both murine and human cells, and *in vivo* in murine models of experimentally induced colitis. Dextran sodium sulfate (DSS) and trinitrobenzene sulfonate (TNBS) are commonly used to induce colitis in mice, by initiating epithelial cell lesions and altering the epithelial barrier integrity. Both acute DSS- and TNBS-induced colitis are characterized by a Th1–Th17-mediated acute inflammation with increased TNF-α, IL-6, IL-17 and increased IL-12, IL-17, IFN-γ respectively. However, while chronic TNBS-induced colitis is associated with an enhanced Th1/Th17 intensity of response, chronic DSS-induced colitis switches to a Th2-biased (IL-4, IL-10) profile. Similarly, histological DSS-colitis features are "UC-like" (epithelial disruption, focal lesions, superficial inflammation), while histological TNBS-colitis features are "CD-like" (transmural inflammation and edema) [120].

The first observed immunomodulatory effect of MSCs was their ability to inhibit T cell proliferation *in vitro* [121–123]. This phenomenon was observed independently of whether the T cells were $CD4^+$ or $CD8^+$, naive or memory T cells [124], stimulated with allogeneic cells or nonspecific mitogens [123], and regardless of their functional state or the type of TCR expressed [125]. The immunosuppressive activity of MSCs is independent of the major histocompatibility complex (MHC), as inhibition is similar using third-party MHC-unmatched MSCs or MSCs that are autologous to the responder or stimulating lymphocytes [121]. MSC-inhibited T cells do not undergo apoptosis [123], but are blocked in the $G_0/G_1$ phase of the cell cycle in a state of anergy by inhibition of cyclin D2 expression [126]. Whether *in vitro* addition of IL-2 can [122] or cannot [127] reverse this inhibition is controversial. However, a recent study found that the systemic infusion of murine BM-MSCs induced T cell apoptosis via the FAS ligand/FAS pathway, and that $FASL^{-/-}$ bone-marrow-derived MSCs (BM-MSCs) could not ameliorate DSS-induced colitis in mice [128]. Interestingly, it has been demonstrated that FAS-mediated apoptosis is lower in the T cells of people with CD than in control T cells [129]. However, another study demonstrated that *in vitro* MSCs prevented activation-induced cell death through downregulation of Fas receptor and Fas ligand on TCR-activated T cells [127]. It is important to note that the effect of MSCs on T cells seems dependent on the MSC/T cell ratio: a high MSC/T cell ratio exerts strong inhibitory effects, while low MSC/T cell ratios enhance T cell proliferation [130].

Other studies suggest that an important part of MSC-mediated immunomodulation results from the recruitment of immune-regulatory T cells (Tregs), which can be either $CD4^+$ $CD25^+$ $FoxP3^+$ or $CD8^+$ from both naive and memory T cells [131–133]. *In vivo*, MSC-mediated Treg expansion has been observed in several immune-mediated diseases, including experimental autoimmune encephalomyelitis [134], experimental arthritis [135], and type 1 diabetes mellitus [136]. Mechanisms of this T-reg expansion appear multiple and include secretion of soluble factors by MSCs, such as prostaglandin $E_2$ ($PGE_2$) and TGF-β1 [137–139] or HLA-G [140,141], interaction between MSCs and chemokine ligand 1 and chemokine receptor 8 on T cells [142], and induction of immature DC [143] and monocytes [144] to a regulatory profile. MSC-mediated T cell modulation was observed in several DSS- and TNBS-induced colitis mouse models, treated with intravenous or intraperitoneal injection of human adipose-derived MSCs (ASCs) [145], umbilical-cord-derived MSCs (UCMSCs) [146,147], or BM-MSCs [148]. In these studies, MSCs effectively treated colitis with both a clinical response and histological improvement, showing decreased infiltration of inflammatory cells in the lamina propria. The investigators mostly observed a decreased number of IFN-γ-producing Th1 cells and higher numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs in mesenteric lymph nodes and the lower colon and systemic levels of Th1 and Th17 proinflammatory cytokines, including TNF-α, IFN-γ, IL6, IL-12, IL-17, and IL-23, as well as higher colonic and systemic levels of the anti-inflammatory/regulatory cytokine IL-10. In a murine model of TNBS-induced colitis, MSCs co-localized with $CD11b^+$ cells in the spleen, and these cells were essential to the expansion of Tregs in draining mesenteric lymph nodes [149]. In another murine model of TNBS-induced colitis, intravenous injection of murine BM-MSCs was followed not only by downregulation of both Th1 and Th17 responses and activation of regulatory T cells, but also by a significant upregulation of Th2 activities (IL-4, IL-10, GATA-3) [150].

The effect of MSCs on B cells remains controversial, and may depend on environmental signals. According to most authors, MSCs inhibit B cell activation, proliferation, chemotaxis, and immunoglobulin G secretion, both in murine [126,151,152] and human [153] studies. In these studies, B cells were enriched from the spleen or purified from peripheral blood and were exposed to various stimuli, including allogeneic cells, mitogenic agents and lipopolysaccharide. Inhibition of B cell function was mediated by soluble factors in transwell experiments. In another study, B-cell co-stimulatory molecule expression and cytokine production seemed unaffected by human MSCs [153]. However, in a study of highly purified B cells, MSCs promoted proliferation and differentiation into immunoglobulin-secreting cells of naive B cells isolated from healthy donors and total B cells from children with the autoimmune disease systemic lupus erythematosus (SLE), stimulated with an agonist of toll-like receptor (TLR) 9 in the absence of B cell receptor triggering [154].

The relationship between MSCs and NK cells is complex and also not yet fully understood. MSCs inhibit the IL-2- or

IL-15-driven proliferation of human resting NK cells and their production of IFN-γ, TNF-α, and IL-10 [155,156], but their effects on the cytotoxic activity of NK cells is controversial. Some authors report have reported MSC-mediated decreased NK cell cytotoxicity [140,156,157], while others have not observed this effect [158,159]. This discrepancy might depend on MSC/NK ratios or on whether NK cells were freshly isolated or not [155].

MSCs also interact with antigen-presenting cells, including DCs. MSCs inhibit differentiation of DC precursors, such as monocytes or $CD34^+$ hematopoietic progenitors into mature DCs, by blocking them in the $G_0/G_1$ phase of the cell cycle, through a downregulation of cyclin D2 and $p27^{Kip1}$ expression [160]. DCs cultured with MSCs have impaired antigen-presenting [161] and migration [162] functions and show increased secretion of IL-10 and decreased production of IL-12 [163]. These effects have been confirmed *in vivo* in murine models [164]. MSCs also induce DC precursors ($CD34^+$ hematopoietic progenitors) [165] and mature DCs [166] to differentiate into a regulatory DC population through the Notch signaling pathway.

MSCs may also modulate the functions of macrophages, for example, through increased IL-10 secretion [167] or induction into a regulatory-like profile [144]. *In vitro* addition of increasing numbers of monocytes or DCs into co-cultures of human ASCs and activated $CD4^+$ cells progressively inhibited T cell proliferation and IFN-γ production [145]. In murine models of DSS- and TNBS-induced colitis, the intraperitoneal injection of murine bone marrow macrophages co-cultured with ASCs was effective in treating both colitis and sepsis. *In vitro* these macrophages showed a regulatory phenotype that was different from activated macrophages and characterized by high arginase activity and increased expression of IL-10 and inducible nitric oxide synthetase (iNOS) upon restimulation with lipopolysaccharide, while secreting low levels of the inflammatory cytokines TNF-α and IL-12. They showed potent immunosuppressive activity on splenocytes that was significantly reversed by the iNOS inhibitor L-NG-nitroarginine methyl ester and IL-10-blocking antibodies [168].

Thus the mechanisms of MSC-mediated immunomodulation are not yet fully elucidated and probably vary among species [169]. They include direct cell-to-cell contact through the expression of adhesion molecules and secretion of soluble factors. Multiple factors are involved, including $PGE_2$ [156,167,170], indoleamine 2,3-dioxygenase [114,156,171], nitric oxide [172,173], HLA-G [140,174], TGF-β and hepatocyte growth factor [123], IL-1 receptor antagonist [175], TNF-stimulated gene 6 protein [176], and IL-10, which, although probably not secreted directly by MSCs, also participates in the immunosuppressive effect of MSCs, as IL-10 blockade partially reversed the colitis and sepsis in the murine model [145]. In the DSS murine models of DSS- or TNBS-induced colitis, $PGE_2$ produced by nucleotide-binding oligomerization domain-containing protein-2-activated human UCMSCs increased production of IL-10 and Tregs and was required to reduce the severity of colitis [177].

Transcriptional repression of the proinflammatory cytokine early T cell activation factor 1 (osteopontin) by the autoimmune regulator (AIRE) has also been implicated in MSC immunomodulation as it was required for the efficacy of BM-MSC administration in a model of chronic colitis by transfer of $CD4^+$ $CD45RB^{hi}$ T cells in $Rag^{-/-}$ mice. However, AIRE did not control MSC suppression of T cell proliferation *in vitro* [178].

The immunomodulatory effects of MSCs depend on the inflammatory environment and in some circumstances MSCs can act as immune-stimulating cells. TLRs have an important role in inducing MSCs into an immunosuppressive or immunostimulating pathway, as different TLR agonists influence MSC proliferation, differentiation, migration, and immunomodulatory functions in different ways [179–181]. Many inflammatory cytokines promote MSC-mediated immunosuppression, including IFN-γ, TNF-α, and IL-1β [173]. Pretreatment of human BM-MSCs with IFN-γ increased expression of MHC class II molecules, indoleamine 2,3-dioxygenase, and iNOS and significantly inhibited peripheral blood mononuclear cell (PBMC) proliferation at lower PBMC : MSC ratios. In murine models of DSS- and TNBS-induced colitis, intraperitoneal administration of INF-γ-pretreated MSCs was more effective in treating colitis, in reducing serum amyloid A levels, in reducing colonic inflammatory cytokines (TNF-α, IL-6, IFN-γ, and IL-17A), and in increasing migration into inflamed intestine [182]. Similarly, IL-1β-primed human UCMSCs significantly attenuated the development of DSS-induced murine colitis, increased the number of peritoneal M2 macrophages and splenic or mesenteric lymph node Tregs, and enabled migration more efficiently into the spleen, mesenteric lymph nodes, and colon, which was related to upregulation of CXCR4 expression. IL-1β stimulation elevated COX-2, IL-6, and IL-8 mRNA expression in MSCs [183].

### 50.5.2 Immune tolerance

Human MSCs are immunoprivileged cells because they express low levels of HLA class I molecules and do not express HLA class II molecules nor the co-stimulatory molecules CD80, CD86, and CD40 under normal circumstances [184,185]. However, expression of HLA class I antigens, even at a low level, may be responsible for vulnerability of MSCs to activated NK-cell lysis [186]. Moreover, a recent study observed the capacity of murine MSCs to present extracellular antigen through their MHC class I molecules by cross-presentation and to induce an effective $CD8^+$ T cell immune response [187]. In addition, it has been demonstrated *in vivo* that, in the presence of a narrow window of IFN-γ, MSCs upregulate expression of MHC class I and II molecules and are able to act as antigen-presenting cells and stimulate $CD4^+$ cell proliferation [188–190]. This immunogenicity has also been observed *in vivo* in animal models in which donor-derived MSCs, unlike autologous MSCs, could promote bone marrow [191] or skin graft [192] rejection. Systemic or local administration of allogeneic MSCs in rats resulted in the development of anti-donor T cell and antibody responses, which could

promote a rapid clearance of MSCs and a limitation of their long-term benefits [193,194].

### 50.5.3 Tissue regeneration

MSCs can differentiate *in vitro* to cells of the mesenchymal lineage. The relative importance of this property for the efficacy of MSCs in experimental colitis and IBD is still being investigated.

### 50.5.4 Homing

MSCs are able to selectively home to sites of tissue injury and inflammation, including intestine, kidney, lung, liver, thymus and skin [195] (and see Chapters 22–24). In murine models of colitis, MSCs have demonstrated their ability to migrate selectively into inflamed zones of the intestine, mesenteric lymph nodes, and spleen, whether they were murine or human, bone marrow, adipose, or umbilical cord derived, and whether administered intravenously or intraperitoneally [145,147–149,196]. MSCs have been found in the lamina propria [196], the muscular layer, and the submucosa of the inflamed intestine [197]. However, homing capacities may be altered in MSCs that have been cryopreserved. In a murine model of TNBS-induced colitis, intraperitoneally but not intravenously injected cryopreserved MSCs were found in the inflamed colon [197]. MSC migration is directed by a multitude of signals depending on the MCS source, type or environment, and growth factors and cytokines involved [198,199]. In order to better understand the selective migration of MSCs into the inflamed intestine, Gonzalez-Rey *et al.* [145] studied the expression of various chemokine receptors involved in cell recruitment to inflammatory sites and demonstrated that human ASCs expressed on their surface the chemokine receptors CCR1, CCR2, CCR4, CCR5, CCR7, CCR9, CXCR1, and CXCR5 and that most of these receptors were functional since human ASCs migrated in response to chemokines such as CCL5, CCL22, CCL19, CCL25, CXCL8, and CXCL13. Moreover, human ASCs expressed a panel of adhesion molecules involved in tissue transmigration, including CD54, CD49e, CD44, CD29, CD105, CD106, and CD166. Activation and targeting may improve delivery of MSCs into mesenteric lymph nodes and colon, and hence their efficacy in murine colitis, as demonstrated with IFN-γ- [182] or IL-1β-primed MSCs [183] and VCAM antibody-coated MSCs (AbVCAM-1-MSCs) [200]. IFN-γ upregulates expression of the chemokine receptor CXCR7, lectins LGALS3BP and LGALS9 and ADAM15, a matrix metalloproteinase involved in epithelial adhesion [182], while IL-1β upregulates CXCR4 expression [183]. How long MSCs do stay into the inflamed intestine is unknown, but it seems that they remain there for at least 2 weeks [147].

### 50.5.5 Differentiation and stimulation of tissue repair

In addition to their capacity to differentiate into chondrocytes, osteoblasts and adipocytes, MSCs can also differentiate into other cells of the mesenchymal lineage, such as myoblasts [201] or cardiomyocytes [202]. Moreover, UCMSCs [203] and BM-MSCs have shown an ability to differentiate *in vitro* into cells of ectodermal lineage, such as neuroglial cells [204,205], and of endodermal lineage, such as renal tubular epithelial cells [206] and hepatocytes [207–209], although this a controversial area; and while MSCs may take on the morphological and even the cell surface markers of, for example, neurons, there is little, if any, evidence that they function as neurons. This not to say that they are not beneficial in the treatment of diseases involving damaged neurons, but they most likely mediate this effect by secretion of paracrine factors and by modulation of the inflammatory reaction.

As indicated earlier, in response to inflammation or tissue injury, MSCs are capable of engrafting in many tissues [210], albeit transiently, and have demonstrated their efficacy in promoting tissue repair in myocardial infarction [211], kidney diseases [212,213], liver diseases [214], lung diseases [215] and injury [216], and neurological disorders [217]. In DSS-induced colitis in rats, intravenous injection of rat MSCs enabled significant healing of epithelial injuries, and the MSCs extracted from the lamina mucosa had upregulated their expression of α-smooth muscle actin, suggesting a reprogramming to myogenic lineage differentiation, possibly involved in intestinal repair. In TNBS-induced colitis in rats, topical implantation of rat BM-MSCs healed mucosal injuries. MSCs engrafted into the submucosal layers, but only a small proportion was positive for α-smooth muscle actin and desmin, indicating differentiation into myofibroblasts. A larger proportion of MSCs surrounding the lesion area expressed vascular endothelial growth factor and TGF-β1, two growth factors known to play important roles in gastrointestinal wound healing [218]. In a DSS-induced colitis model in rats, intravenous injection of allogeneic MSCs resulted in clinical improvement with markedly reduced epithelial injury and, significantly, restoration of expression of claudin-2, -12 and -15, proteins constituting tight junctions that play a major role in the paracellular permeability of the epithelial barrier [219].

Again, as indicated earlier, it is thought that the tissue repair properties of MSCs are mainly due to their ability to stimulate survival and recovery of local tissues, rather than to transdifferentiation ability [220]. MSCs have demonstrated their ability to stimulate angiogenesis [221] and to inhibit apoptosis and fibrosis in injured tissues [222].

## 50.6 Mesenchymal stromal cell administration in inflammatory bowel diseases

Two different applications of MSCs in IBDs have been evaluated in human trials: local injection of MSCs to treat fistulizing CD and intravenous injection of MSCs to treat luminal CD or UC. MSC administration has been studied in CD more than it has in UC.

### 50.6.1 Mesenchymal stromal cell administration for fistulizing Crohn's disease

Management of fistulas in patients with CD is an extremely challenging problem, because many such fistulas do not respond to available treatment, despite recent improvements using anti-TNF agents and improvements in surgical treatments. Endoanal advancement flap is the standard surgical technique, but anal incontinence and recurrence are not uncommon. Several trials have evaluated the efficacy of local injections of ASCs or BM-MSCs.

### 50.6.2 Autologous mesenchymal stromal cell administration for fistulizing Crohn's disease

#### 50.6.2.1 Adipose-tissue-derived mesenchymal stromal cells

In a phase I study García-Olmo *et al.* treated four patients with one or more refractory, complex Crohn's fistulas with a single intrafistular injection of MSCs. The fistulas were enterocutaneous, suprasphincteric, and/or rectovaginal fistulas, were unresponsive to medical treatment, and had been unsuccessfully treated by surgery at least twice. The injection consisted of $3–30 \times 10^6$ autologous ASCs from passages 1 to 3. Six of eight (75%) fistulas had healed 8 weeks after treatment, and the other two (25%) were incompletely closed with decreased output flow. No relationship was found between the number of injected MSCs and the success of the treatment. No adverse effects were observed within 12–22 months follow-up, and no aberrant differentiation was seen in biopsies [223].

In a phase IIb study the same group showed that ASCs were effective in healing 69% of complex perianal fistulas in patients with moderate to severe CD who were intolerant or resistant to the anti-TNF agent infliximab [224]. Forty-nine patients with complex perianal fistulas were included. Thirty-five of the fistulas were of cryptoglandular origin and 14 were associated with CD. Cryptoglandular anal fistulas arise from inflammation of the proctodeal glands of the intersphincteric space. The mucus secretions of the anal glands empty into the anal crypts, thereby lubricating the anus. Anal glands are present in the subepithelium and the internal sphincter, with a large number also deeply sited within the intersphincteric space. Infection of these intersphincteric glands is thought to give rise to an intersphincteric abscess if the draining duct is blocked by the resulting infectious debris. The abscess may resolve by spontaneous drainage into the anal canal or it may progress to an acute anorectal abscess that, in most cases, subsequently develops into a perianal fistula. This type of fistula is not associated with CD.

Patients were randomly assigned to the administration of an intrafistular injection of fibrin glue or fibrin glue containing 20 million ASCs. If fistula healing had not occurred by 8 weeks, a second double dose of ASCs was administered. Fistula healing was observed in 17/24 patients receiving ASCs (71%); 11 patients healed after the first injection and six patients healed after a second injection compared with 4/25 (16%) in the control group (RR 4.43, $P < 0.001$). A similar proportion was observed in the CD patients, with healing of the fistula in five out of seven patients receiving ASCs compared with one out of seven patients in the control group (RR 5.00, $P = 0.10$). Improvement of quality of life, assessed by a quality-of-life questionnaire, was significantly greater in the ASC group compared with the control group, even in patients whose fistula did not heal [225]. After an average long-term follow-up of approximately 40 months, 7 out of 12 patients treated with ASCs remained free of recurrence compared with two out of three in the control group. Perianal sepsis occurred more frequently in the control group than in the ASC group ($P = 0.04$). No adverse effects related to ASC administration were noted, confirming their excellent safety and tolerability profile, and no patient suffered from anal incontinence [226].

Another group performed a dose-escalation phase I study, in which 10 patients with a perianal fistula associated with CD were treated with an intrafistular injection of autologous ASCs from passages 3 and 4 at a dose of either $1 \times 10^7$ (group 1), $2 \times 10^7$ (group 2), or $4 \times 10^7$ ASCs/mL (group 3). The doses used were selected with regard to the size of the fistula. Eight weeks after injection, complete healing occurred in two out of four patients in group 2 and one out of three patients in group 3, while partial closure with no output drainage was observed in all other patients. After follow-up of 8 months the three patients whose fistula had been completely healed were free of recurrence and no adverse effects were observed [227]. The same group subsequently led a phase II trial studying the effects of the intrafistular injection of ASCs from passages 3 and 4 in 43 patients with perianal fistulas associated with CD. The first injection again contained a number of ASCs that was proportional to the size of the fistula: $3 \times 10^7$ ASCs per 1 cm length of fistula when the fistular diameter was less than 1 cm, and $6 \times 10^7$ ASCs per 1 cm length of fistula when the diameter was between 1 and 2 cm. The average number of ASCs was $15.8 \times 10^7$ cells per fistula. Patients without complete fistula closure at 8 weeks received a second injection of 1.5 times more cells than the first injection. The fistula tract was filled with a mixture of ASCs and fibrin glue. Complete fistula healing was observed in 27/42 patients (64.3%) by 8 weeks after the final ASC injection and was sustained after 1 year in 23/26 patients (88%). Six other patients showed incomplete closure of the fistula, of which five patients achieved closure of more than 50% of the fistula tract with a decrease in drainage of more than 50%. Five other patients discontinued the study before week 8. No relation was found between the outcome of the treatment and fistula type, length, or diameter or duration of CD. No adverse events related to ASCs were observed [228].

#### 50.6.2.2 Bone marrow-derived mesenchymal stromal cells

The safety and efficacy of serial intrafistular injections of autologous BM-MSCs in the treatment of fistulizing CD was studied by

Ciccocioppo *et al.* in 10 patients refractory to all previous medical treatments including anti-TNF agents or unsuccessfully treated by surgery. They administered intrafistular injections of $15–30 \times 10^6$ MSC from passage 3 every 4 weeks until improvement was obtained or when autologous MSCs were no longer available Patients received between two and five injections. Sustained complete closure of the fistula tract occurred in 7/10 patients and incomplete closure in 3/10 patients as assessed by surgical exploration and MRI. MRI provided images of regenerative tissue without fibrotic tissue. All patients had a significant reduction of their CDAI scores: pre- and posttreatment median values were $294 \pm 49$ and $99 \pm 32$ at 12 months after the last procedure ($P < 0.001$). Their median PDAI scores pre- and posttreatment were $13.0 \pm 2.2$ and $4.5 \pm 2.4$ at 12 months after the last procedure ($P < 0.001$). Patients who achieved remission had CDAI scores ≤150 and PDAI scores ≤8, usually after the second administration of ASCs. Lower endoscopic examination performed at the end of the follow-up period demonstrated rectal mucosal healing in seven out of seven patients. In addition, they observed a significant increase in the percentage of circulating $CD4^+$ $CD25^{bright}$ $FoxP3^+$ Tregs as soon as after the second injection of ASCs which was stable at 12 months ($P < 0.01$). Additionally, mucosal $FoxP3^+$ Tregs were present in the inflamed areas at 12 months ($P < 0.0001$). There were no adverse effects during the procedure nor during the 12-month follow-up period [229].

### 50.6.3 Allogeneic mesenchymal stromal cell administration for fistulizing Crohn's disease

García-Olmo and co-workers also explored the use of expanded allogeneic ASCs for the treatment of complex perianal fistulas in patients with nonactive luminal CD (CDAI ≤200) in a single-arm multicenter phase I/IIa clinical trial. Twenty-four patients received an intrafistular treatment with $20 \times 10^6$ allogeneic ASCs followed by a second administration of $40 \times 10^6$ ASCs if fistula closure was incomplete after 12 weeks. After 12 weeks, 12/20 patients (60.0%) had an improvement of at least one of their fistulas and complete closure occurred in 8/21 (38.1%) on clinical examination. After 24 weeks, these values were respectively 9/13 patients (69.2 %) and 9/16 patients (56.3%). Based on a combination of clinical and MRI assessment, fistula closure was seen in 6/24 patients (30%) at 24 weeks. A statistically significant decrease in the MRI score of severity was observed at week 12. PDAI significantly decreased by more than 37% compared with baseline ($P < 0.01$). No effect was observed on the CDAI scores. Luminal relapse was observed in 0/24 patients (0.0%) at week 12 and in 5/24 patients (20.8%) at week 24. After a 6-month follow-up period the safety profile was acceptable, with a low number of serious adverse events: anal abscess in three patients, pyrexia in one patient, and uterine leiomyoma in one patient [230].

### 50.6.4 Autologous mesenchymal stromal cell administration for luminal inflammatory bowel diseases

Intravenous injections of autologous BM-MSCs have been tested in a phase I trial. Nine adult patients with moderate to severe CD with a CDAI score between 220 and 450 and who were refractory to conventional standard treatment, including corticosteroids, immunosuppressive agents, or anti-TNF therapy, were treated with two intravenous injection of $1–2 \times 10^6$ cells/kg body weight 7 days apart. Three patients had a clinical response (defined as a decrease in CDAI score of ≥70 points) but none achieved remission (CDAI <150), and four patients experienced worsening of their disease, requiring surgery in three cases or rescue medication in one case within 14 weeks after BM-MSC administration. Endoscopic improvement using the SESCD was seen in two patients at week 6. Biopsies of inflamed mucosa showed a trend to lower $CD4^+$ T cell and higher $CD4^+$ $CD127^+$ Treg numbers [231]. CD127 is the receptor for IL-7.

### 50.6.5 Allogeneic mesenchymal stromal cell administration for luminal inflammatory bowel diseases

Intravenous injection of allogeneic BM-MSCs has also been evaluated with more success in a phase II pilot study. Nine patients with refractory moderate-to-severe CD with a CDAI score ≥220 and previously unsuccessful treatment with corticosteroids and immunomodulatory agents were randomized to receive either a low ($2 \times 10^6$ BM-MSCs/kg) or a high ($8 \times 10^6$ BM-MSCs/kg) dose of MSCs by intravenous infusion. After 28 days all patients had a decrease in their CDAI score, with a mean decrease of 105 ($P = 0.004$), and three patients had a clinical response (reduction in CDAI ≥100), one of whom entered clinical remission (CDAI <150). Patients reported an increased quality of life, as attested by a significant increase in mean Inflammatory Bowel Disease Questionnaire (IBDQ) score by day 28 ($P = 0.008$). No infusion reaction was observed, although five patients experienced mild to moderate adverse events [232].

The Nanjing group, who studied the use of MSCs in the autoimmune disease SLE, reported their experience of allogeneic MSC transplantation in seven patients with IBD, among whom four had CD and three had UC. They administered one intravenous infusion of BM-MSCs from healthy family members or UCMSCs at a dose of $1 \times 10^6$/kg body weight. Patients continued their treatment with corticosteroids and/or immunosuppressive agents after the transplant. After 3 months they observed a significant reduction in disease activity (CDAI or Clinical Activity Index) in all patients, with remission achieved in five out of seven patients (two out of four with CD and three out of three with UC) and endoscopic improvement with a decrease in Endoscopic Index of Severity score or in Endoscopic Activity Index in three out of seven patients, two out of four with CD and one out of three with UC. Remission lasted for more than 2 years in two patients, while two other patients relapsed at 6 and

7 months. One CD patient had a significant reduction in fistula size and drainage. Histological analysis of biopsy specimens showed a reduction in the extent of the inflamed area and in the lymphocytic infiltration in the mucosa propria. One patient had a significant reduction in fistula size and drainage. No serious adverse events were reported after a mean follow-up of 19 months (range 6–32 months) [233].

## 50.7 The future of mesenchymal stromal cell treatment in inflammatory bowel diseases

### 50.7.1 Ongoing protocols

#### 50.7.1.1 Mesenchymal stromal cell treatment for fistulizing Crohn's disease

##### *50.7.1.1.1 The Navarra study (NCT01157650)*

This phase I–II study is evaluating viability, safety, and tolerance of locally implanted autologous ASCs in 15 patients with non-active CD (CDAI ≤200) and one or more enterocutaneous, rectovaginal or complex perianal fistulas. Patients should not have perianal abscesses or rectal and/or anal stenosis, and should not have received anti-TNF therapy or have received tacrolimus or cyclosporine within 8 weeks and 4 weeks before MSC therapy respectively. Secondary outcomes include an evaluation of efficacy (fistula healing, quality of life, presence of systemic CD, relapse) and a biological characterization of the MSCs used in terms of their phenotype, immune suppressive capacities, and cytokine production. This study is ongoing, but is no longer recruiting participants (Figure 50.8).

##### *50.7.1.1.2 The Leiden study (NCT01144962)*

This dose-escalation phase I–II study aims to determine the safety and efficacy of intrafistular injection of allogeneic BM-MSCs for the induction of response of active fistulizing CD. The fistulas are peri-anal fistulas refractory to conventional medical treatment. Patients are randomized into one of four groups, receiving either placebo or MSCs at doses of 10 million, 30 million, or 90 million. Exclusion criteria include acute peri-anal infection, rectovaginal or complex peri-anal fistulas with more than two internal openings, and anti-TNF therapy less than 8 weeks prior to enrolment. Secondary outcomes at 12 weeks are improvement of clinical and endoscopic scores, quality of life details, and serum CRP. Safety is evaluated after 12 and 24 weeks. This study is ongoing, but is no longer recruiting participants.

##### *50.7.1.1.3 The Anterogen studies (NCT01314092, NCT01623453)*

In a phase II trial, patients with complex perianal fistulas are randomized to receive autologous ASCs at either low ($1 \times 10^9$/mL) or high ($2 \times 10^9$/mL) dose, followed by a second double-dose injection if complete closure is not achieved. Primary outcome is complete closure of the fistula at week 8. Safety is assessed by the reporting of all adverse events. Subsequently, an open follow-up clinical trial will evaluate the number of patients with sustained complete closure and the number of adverse events 6 months after the final dose injection.

##### *50.7.1.1.4 The TiGenix study (NCT01541579)*

This phase III randomized double-blind placebo-controlled multicenter study is evaluating the efficacy of allogeneic ASCs for the treatment of complex anal fistulas in patients with CD. Patients with nonactive or mildly active luminal CD (CDAI ≤220) and two or less complex perianal fistulas are randomized to receive either an intrafistular injection of $120 \times 10^6$ allogeneic ASCs or placebo. Patients with abscesses, rectovaginal fistulas, surgically treated fistulas, and ongoing corticosteroid treatment or corticosteroid within the preceding 4 weeks are excluded from

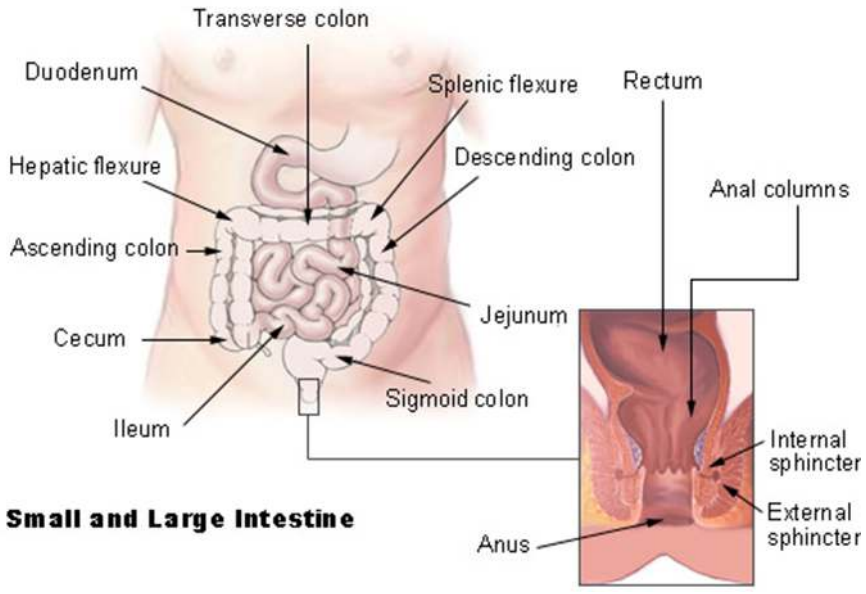



**Figure 50.8** The small and large intestine with an insert of the perianal anatomy, an area where perianal fistulas occur in persons with CD. *Source:* http://commons.wikimedia.org/wiki/File:Illu_intestine.jpg#mediaviewer/File:Illu_intestine.jpg.

the study. Primary outcomes include remission of perianal fistulizing CD at week 24 by clinical and MRI assessment. Other outcomes are efficacy, including response, time to response, and time to remission, and assessment using the PDAI and IBDQ criteria, as well as safety at weeks 24 and 52. This study is currently recruiting participants.

#### *50.7.1.1.5 The Royan study (NCT01874015)*

This study is a prospective, randomized, phase I trial in which patients with refractory CD, a CDAI score >220, and perineal fistulas are randomized to receive four monthly intrafistular injections of autologous BM-MSCs with or without fibroblasts. Outcomes are fistula closure and CDAI score at month 4. This study is currently recruiting participants.

### 50.7.1.2 Mesenchymal stromal cell administration for luminal inflammatory bowel diseases

#### *50.7.1.2.1 The Liège study (NCT01540292)*

This prospective open-label phase I–II pilot trial was initiated in 2013 to explore the safety and efficacy of allogeneic BM-MSC infusions in CD refractory or intolerant to conventional therapies. Twenty patients with active refractory CD defined by a CDAI >220 despite conventional treatment, including mesalazine, corticosteroids, purine analogues, methotrexate, infliximab, and adalimumab, are treated with two successive injections of $1.5–2.0 \times 10^6$ allogeneic BM-MSCs/kg at the beginning of the trial and 4 weeks later. Conventional treatments of CD, such as mesalazine, methylprednisolone, budesonide, azathioprine, 6-mercaptopurine, or methotrexate, are allowed, while anti-TNF antibodies are forbidden, and antibiotics are allowed only for the treatment of a concurrent infection. The primary endpoint is a clinical response defined by a 100-point decrease in the CDAI score at week 8. Secondary endpoints include clinical response, remission defined by a CDAI score <150, CDAI levels, CRP levels, and fecal calprotectin levels at weeks 2, 4, 8, and 12. Immune modulation is being investigated by monitoring of nucleated cell counts, leucocyte subpopulations, Treg numbers, immunoglobulin levels, Vβ repertoire of T lymphocytes, and the quantification of TRECS in T lymphocytes. TRECS are TCR excision circles, which are small circles of DNA created in T cells during their passage through the thymus as they rearrange their TCR genes. This is a measure of thymic function. Safety is being assessed by recording side effects, including infections.

#### *50.7.1.2.2 The Mesoblast international studies (NCT01233960 and NCT00543374)*

In a first phase III study, patients with moderate-to-severe CD (CDAI scores between 250 and 450) who are intolerant to, or who have previously failed, therapy with at least one corticosteroid course and at least one immunosuppressive agent course and an anti-TNF treatment are randomized into three groups to receive a placebo or an intravenous injection of allogeneic MSCs at low ($6 \times 10^6$ MSCs) or high ($1.2 \times 10^9$) doses on four occasions over 2 weeks. The primary outcome is disease remission (CDAI score ≤150) at day 28, and secondary outcomes are disease improvement (reduction in CDAI score of ≥100 points), improvement in quality of life (using the IBDQ), and reduction in number of draining fistulas. In a second phase III study, patients who successfully achieved clinical benefit in the first study will be enrolled and followed for duration and reinduction of clinical benefit using the CDAI questionnaire and improvement in quality of life using the IBDQ after 6 months.

#### *50.7.1.2.3 The Qingdao study (NCT01221428)*

This phase I–II study aims to determine the safety and efficacy of UCMSCs in refractory UC. First, $2 \times 10^7$ MSCs are injected intravenously followed 1 week later by $1 \times 10^7$ MSCs injected into the mesenteric artery. Outcomes evaluated after 3 months are improvement in endoscopy findings and histology and amelioration of clinical symptoms.

#### *50.7.1.2.4 The La Paz study (NCT01914887)*

In this phase I–II study, patients with left-sided colitis and moderate activity as assessed using a modified Truelove–Witts score between 11 and 21, who did not respond to 4 weeks of treatment with oral and/or topical 5-aminosalicylates, receive multiple endoscopic injections of allogeneic ASCs into the affected colonic submucosa at a total dose of $6 \times 10^7$ MSCs. Safety and efficacy assessed by the modified Truelove–Witts score, IBDQ, Mayo endoscopic index, CRP, and fecal calprotectin are evaluated at weeks 0, 4, 8, and 12. The study is currently recruiting.

#### *50.7.1.2.5 The Washington study (NCT02150551)*

In this phase I trial, pediatric patients with IBDs will be randomized into three dose groups and will receive eight weekly infusions of allogeneic BM-MSCs. Safety and efficacy will be assessed using the pediatric CDAI or Pediatric Ulcerative Colitis Activity Index questionnaires, quality of life using the Impact III IBD questionnaire, laboratory tests, including CRP, fecal calprotectin, and proinflammatory cytokines, as well as endoscopic healing evaluated at day 24 and day 77.

## 50.8 Issues to be resolved

### 50.8.1 Source of mesenchymal stromal cells

Most of the published human trials in people with CD have used ASCs. Only Ciccocioppo *et al.* used BM-MSCs [229]. ASCs have the advantage of being easily accessible in large numbers. MSC treatment requires a large number of cells, and, although MSCs have great proliferation potential, extended *ex vivo* expansion can alter their properties, resulting in reduced differentiation potential and senescence from the sixth passage onward [234].

Alternatively, expanded ASCs could be superior to nonexpanded ASCs, known as the stromal vascular fraction, for the treatment of enterocutaneous fistula in CD, as shown by García-Olmo *et al.* in eight patients: three of four fistulas healed in the ASC group compared to one of four in the stromal vascular

fraction group [235]. We lack information on differences on immunophenotype, differentiation potential, transcriptome, and proteome between BM-MSCs and ASCs. *In vitro* studies suggest that ASCs might be superior to BM-MSCs in suppressing immune responses [236,237]. In practice, both types of cells have demonstrated some efficacy, but formal studies comparing the efficacy of BM-MSCs and ASCs will be needed to resolve this question.

### 50.8.2 Autologous versus allogeneic mesenchymal stromal cells

The use of autologous MSCs has raised a debate on whether MSCs are affected by, or may contribute to, CD. MSCs from patients with other autoimmune diseases, such as SLE, have lower proliferation capacities [238], and the results in human studies on SLE were disappointing when autologous MSCs were used. However, several *in vitro* studies have demonstrated that MSCs from patients with CD have similar growth potential and T cell suppression properties to MSCs from healthy donors [231,239]. Moreover, both autologous and allogeneic MSCs seem promising in human trials, with the exception of a trial using autologous MSCs in SLE.

### 50.8.3 Dosage and modalities of administration

Because of the extent and dissemination of luminal lesions in CD, systemic administration seems required. In mice, intravenous and intraperitoneal routes of administration have been used with equal success, apart from a few studies in which MSCs did not appear to home to inflamed intestine [196,218]. In humans, intravenous infusion of cells is simple, minimally invasive, routinely performed, and safe. However, little is known about the optimal amount of MSCs and the proportion of MSCs that will reach an inflamed intestine. Duijvestein *et al.* used $1–2 \times 10^6$ autologous MSCs/kg body without much success [231], while Onken *et al.* observed no significant difference between low ($2 \times 10^6$ MSCs/kg) and high ($8 \times 10^6$ MSCs/kg) [232]. Administration of MSCs via selective mesenteric artery cannulation has been successfully performed in one patient, without any complication [240]. This route of administration may increase the number of MSCs reaching the inflamed organ, but its superiority over the intravenous administration is yet to be demonstrated.

Local injection of MSCs has been successfully used in several studies to treat CD fistulas. In studies showing efficacy of intrafistular injections, $10–60 \times 10^6$ MSCs were injected into each fistulous tract, and these injections were sometimes repeated. Ongoing trials are testing higher systemic doses: the Mesoblast international studies are testing the injection of $6 \times 10^8$ or $1.2 \times 10^9$ cells four times over a 2-week period. Several local dose-escalation trials are also currently ongoing.

### 50.8.4 Concomitant use of other drugs

MSCs are used concomitantly with immunosuppressive drugs, and, as they have common targets, it is important to know if the drugs can affect MSC function. MSCs exposed to physiological concentrations of azathioprine, methotrexate, 6-mercaptopurine, and anti-TNF-α antibodies had normal survival and inhibitory capacities on PBMC proliferation when tested *in vitro*. An additive effect could even exist with 6-mercaptopurine and anti-TNF-α antibodies [241].

## 50.9 Safety

So far there have been no reports of any serious adverse events or ectopic tissue growth in clinical trials using MSC-based therapy for CD, nor for graft-versus-host disease [242–244], solid-organ transplantation [245], cartilage disorders [246], or in many completed clinical trials for a variety of applications [247,248]. In some studies there was a mild and transient fever shortly after the time of administration, and this association was found to be statistically significant in a meta-analysis [248]. More experience is needed in order to confirm the long-term safety of MSCs.

The immunosuppressive properties of MSCs could theoretically increase susceptibility to infections and cancers. However, no infections or malignant diseases have been reported as serious adverse events in MSC-treated CD patients, and there is no evidence of any increased risk in other applications [248]. In the context of solid organ transplantation with MSC-based immunosuppression, one group has even observed a decreased infection risk compared with standard immunosuppressive drugs [245]. Another reason for concern for an increased risk of malignant disease is the need to expand MSCs *in vitro* in order to obtain a sufficient number of cells for MSC-based therapy. While murine MSCs have shown a potential for the development of chromosomal aberrations and tumor generation after long-term *in vitro* culture [249,250], human MSCs have not shown any of these risks so far [251–253]. Moreover, IBDs are associated with a higher risk of developing colorectal cancer. In a murine model of colitis-associated tumorigenesis induced by azoxymethane and DSS, MSC-treated animals showed a significant reduction in tumor number and tumor load compared with control mice, while tumor size remained comparable. This could be linked to a decreased expression of proinflammatory cytokines and down-regulation of STAT3 phosphorylation [254].

Finally, the effects of MSCs on preexisting cancers have also been studied. In animal studies some authors found that MSCs could promote tumor growth [255,256], while others observed a tumor-suppressive activity of MSCs [257–259]. This dual effect could be explained by a context-dependent role of MSCs in regulating tumor growth [260,261]. In a small human trial of 25 patients, co-transplantation of MSCs and HSCs was associated with a higher relapse rate compared with control patients (60% versus 20%). However, this observation has not been confirmed by other studies [262].

## 50.10 Conclusions

MSCs represent a promising therapy for IBD, especially for CD. They probably exert their effects through a combination of

immunomodulation, myogenic differentiation, and the promotion of epithelial repair. Both local injection of MSCs for fistulizing CD and intravenous injection of MSCs for luminal IBD have shown interesting results in several human trials. Unlike current surgical strategies, MSC administration is a minimally invasive procedure, which does not injure the anal sphincter and even promotes a nonfibrotic reparative process of fistulas, with no cases of incontinence reported after MSC treatment. MSCs seem superior to conventional drugs in term of side effects, such as opportunist infections. There are still many questions to answer concerning the optimal source of MSCs, as well as dosage and routes of administration.

The efficacy of MSCs compared with conventional treatments still needs to be demonstrated in randomized controlled trials, several studies of which are ongoing. They will also likely help us in the understanding and use of MSCs to treat IBDs.

## References


1 E. V. Loftus Jr. Clinical epidemiology of inflammatory bowel disease: incidence, prevalence, and environmental influences. *Gastroenterology* 2004;**126**:1504–1517.

2 A. Dignass, R. Eliakim, F. Magro, *et al.* Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 1: definitions and diagnosis. *J Crohns Colitis* 2012;**6**:965–990.

3 C. G. Su, T. A. Judge, G. R. Lichtenstein. Extraintestinal manifestations of inflammatory bowel disease. *Gastroenterol Clin North Am* 2002;**31**:307–327.

4 G. Van Assche, A. Dignass, J. Panes, *et al.* The second European evidence-based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *J Crohns Colitis* 2010;**4**:7–27.

5 A. Franke, D.P. McGovern, J. C. Barrett, *et al.* Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet* 2010;**42**:1118–1125.

6 J. Cosnes, C. Gower-Rousseau, P. Seksik, A. Cortot. Epidemiology and natural history of inflammatory bowel diseases. *Gastroenterology* 2011;**140**:1785–1794.

7 B. Chassaing, A. Darfeuille-Michaud. The commensal microbiota and enteropathogens in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011;**140**:1720–1728.

8 T. T. MacDonald, I. Monteleone, M.C. Fantini, G. Monteleone. Regulation of homeostasis and inflammation in the intestine. *Gastroenterology* 2011;**140**:1768–1775.

9 K. Ina, J. Itoh, K. Fukushima, *et al.* Resistance of Crohn's disease T cells to multiple apoptotic signals is associated with a Bcl-2/Bax mucosal imbalance. *J Immunol* 1999;**163**:1081–1090.

10 J. Itoh, C. de La Motte, S. A. Strong, *et al.* Decreased Bax expression by mucosal T cells favours resistance to apoptosis in Crohn's disease. *Gut* 2001;**49**:35–41.

11 W. Strober, I. J. Fuss. Proinflammatory cytokines in the pathogenesis of inflammatory bowel diseases. *Gastroenterology* 2011;**140**:1756–1767.

12 S. I. Siakavellas, G. Bamias. Role of the IL-23/IL-17 axis in Crohn's disease. *Discov Med* 2012;**14**:253–262.

13 S. Bandzar, S. Gupta, M. O. Platt. Crohn's disease: a review of treatment options and current research. *Cell Immunol* 2013;**286**:45–52.

14 M. F. Neurath, I. Fuss, B. L. Kelsall, *et al.* Antibodies to interleukin 12 abrogate established experimental colitis in mice. *J Exp Med* 1995;**182**:1281–1290.

15 Z. Liu, K. Beboes, H. Heremans, *et al.* Role of interleukin-12 in the induction of mucosal inflammation and abrogation of regulatory T cell function in chronic experimental colitis. *Eur J Immunol* 2001;**31**:1550–1560.

16 I.J. Fuss, C. Becker, Z. Yang, *et al.* Both IL-12p70 and IL-23 are synthesized during active Crohn's disease and are down-regulated by treatment with anti-IL-12 p40 monoclonal antibody. *Inflamm Bowel Dis* 2006;**12**:9–15.

17 A. L. Hart, H. O. Al-Hassi, R. J. Rigby, *et al.* Characteristics of intestinal dendritic cells in inflammatory bowel diseases. *Gastroenterology* 2005;**129**:50–65.

18 K. Matsuaka, N. Inoue, T. Sato, *et al.* T-bet upregulation and subsequent interleukin 12 stimulation are essential for induction of Th1 mediated Immunopathology in Crohn's disease. *Gut* 2004;**53**:1303–1308.

19 B. Oppman, R. Lesley, B. Blom, *et al.* Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000;**13**:715–725.

20 S. Aggarwal, N. Ghilardi, M. H. Xie, *et al.* Interleukin-23 promotes a distinct CD4 T cell activation state characterized by the production of interleukin-17. *J Biol Chem* 2003;**278**:1910–1914.

21 D. Yen, J. Cheung, H. Scheerens, *et al.* IL-23 is essential for T cell-mediated colitis and promotes inflammation via IL-17 and IL-6. *J Clin Invest* 2006;**116**:1310–1316.

22 Y. Chen, C. L. Langrish, B. McKenzie, *et al.* Anti-IL-23 therapy inhibits multiple inflammatory pathways and ameliorates autoimmune encephalomyelitis. *J Clin Invest* 2006;**116**:1317–1326.

23 S. Hue, P. Ahern, S. Buonocore, *et al.* Interleukin-23 drives innate and T cell-mediated intestinal inflammation. *J Exp Med* 2006;**203**:2473–2483.

24 M. C. Kullberg, D. Jankovic, C. G. Feng, *et al.* IL-23 plays a key role in *Helicobacter hepaticus*-induced T cell-dependent colitis. *J Exp Med* 2006;**203**:2485–2494.

25 T. ten Hove, C. van Montfrans, M. P. Peppelenbosch, A. J. van Deventer. Infliximab treatment induces apoptosis of lamina propria T lymphocytes in Crohn's disease. *Gut* 2002;**50**:206–211.

26 J. M. Van den Brande, H. Braat, G. R. van den Brink, *et al.* Infliximab but not etanercept induces apoptosis in lamina propria T-lymphocytes from patients with Crohn's disease. *Gastroenterology* 2003;**124**:1774–1785.

27 W. J. Sandborn, S. B. Hanauer, S. Katz, *et al.* Etanercept for active Crohn's disease: a randomized, double-blind, placebo-controlled trial. *Gastroenterology* 2001;**121**:1088–1094.

28 A. Nesbitt, G. Fossati, M. Bergin, *et al.* Mechanism of action of certolizumab pegol (CDP870): *in vitro* comparison with other anti-tumor necrosis factor alpha agents. *Inflamm Bowel Dis* 2007;**13**:1323–1332.

29 S. Schreiber, M. Khaliq-Kareemi, I. C. Lawrance, *et al.* Maintenance therapy with certolizumab pegol for Crohn's disease. *N Engl J Med* 2007;**357**:239–250.

30 S. Schreiber, P. Rutgeerts, R. N. Fedorak, *et al.* A randomized, placebo-controlled trial of certolizumab pegol (CDP870) for treatment of Crohn's disease. *Gastroenterology* 2005;**129**:807–818.

31 J. Luna, M. C. Masamunt, I. C. Lawrance, M. Sans. Mesenchymal cell proliferation and programmed cell death: key players in fibrogenesis and new targets for therapeutic intervention. *Am J Physiol Gastrointest Liver Physiol* 2011;**300**:G703–G708.

32 W. R. Best, J. M. Becktel, J. W. Singleton, F. Kern Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976;**70** (3):439–444.

33 G. R. Lichtenstein, S. B. Hanauer, W. J. Sandborn, *et al.* Management of Crohn's disease in adults. *Am J Gastroenterol* 2009;**104**: 465–483.

34 W. J. Sandborn, B. G. Feagan, S. B. Hanauer, *et al.* A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 2002;**122**: 512–530.

35 M. Daperno, G. D'Haens, G. Van Assche, *et al.* Development and validation of a new, simplified endoscopic activity score for Crohn's disease: the SES-CD. *Gastrointest Endosc* 2004;**60**:505–512.

36 A. Dignass, G. Van Assche, J. O. Lindsay, *et al.* The second European evidence-based consensus on the diagnosis and management of Crohn's disease: Current management. *J Crohns Colitis* 2010;**4**:28–62.

37 D. G. Binion, G. A. West, K. Ina, *et al.* Enhanced leukocyte binding by intestinal microvascular endothelial cells in inflammatory bowel disease. *Gastroenterology* 1997;**112**:1895–1907.

38 W. J. Sandborn, J. F. Colombel, R. Enns, *et al.* Natalizumab induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2005;**353**:1912–1925.

39 S. R. Targan, B. G. Feagan, R. N. Fedorak, *et al.* Natalizumab for the treatment of active Crohn's disease: results of the ENCORE trial. *Gastroenterology* 2007;**132**:1672–1683.

40 A. C. Ford, W. J. Sandborn, K. J. Khan, *et al.* Efficacy of biological therapies in inflammatory bowel disease: systematic review and meta-analysis. *Am J Gastroenterol* 2011;**106**:644–659.

41 W. J. Sandborn, B. G. Feagan, P. Rutgeerts, *et al.* Vedolizumab as induction and maintenance therapy for Crohn's disease. *N Engl J Med* 2013;**369**:711–721.

42 S. Keshav, D. Johnson, T. Schall, P. Bekker. Chemokine receptor antagonist CCX282-B (Traficet-En™) maintained remission of Crohn's disease in PROTECT-1 study. *Gastroenterology* 2010; **138**:S86.

43 D. W. Hommes, S. R. Targan, A. Dignass, *et al.* Visilizumab therapy in subjects with moderate-to-severe, refractory Crohn's disease. *Gastroenterology* 2007;**132**:A157.

44 D. C. Baumgart, J. N. Lowder, S. R. Targan, *et al.* Transient cytokine-induced liver injury following administration of the humanized anti-CD3 antibody visilizumab (HuM291) in Crohn's disease. *Am J Gastroenterol* 2009;**104**:868–876.

45 S. B. Hanauer, W. J. Sandborn, B. E. Sands, *et al.* A randomized placebo-controlled trial of abatacept for moderately-to-severely active Crohn's disease (CD). *Gastroenterology* 2010; **138**:S86.

46 W. Strober, F. Zhang, A. Kitani, *et al.* Proinflammatory cytokines underlying the inflammation of Crohn's disease. *Curr Opin Gastroenterol* 2010;**26**:310–317.

47 W. J. Sandborn, C. Gasink, L. L. Gao, *et al.* Ustekinumab induction and maintenance therapy in refractory Crohn's disease. *N Engl J Med* 2012;**367**:1519–1528.

48 M. Yamamoto, K. Yoshizaki, T. Kishimoto, H. Ito. IL-6 is required for the development of Th1 cell-mediated murine colitis. *J Immunol* 2000;**164**:4878–4882.

49 R. Atreya, J. Mudter, S. Finotto, *et al.* Blockade of interleukin 6 trans signaling suppresses T cell resistance against apoptosis in chronic intestinal inflammation: evidence in Crohn disease and experimental colitis *in vivo*. *Nat Med* 2000;**6**:583–588.

50 H. Ito, M. Takazoe, Y. Fukuda, *et al.* A pilot randomized trial of a human anti-interleukin-6 receptor monoclonal antibody in active Crohn's disease. *Gastroenterology* 2004;**126**:989–996.

51 M. Lazzerini, S. Martelossi, G. Magazzù, *et al.* Effect of thalidomide on clinical remission in children and adolescents with refractory Crohn disease: a randomized clinical trial. *JAMA* 2013;**310**: 2164–2173.

52 W. Hueber, B. E. Sands, S. Lewitzky, *et al.* Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 2012;**61**:1693–1700.

53 S. R. Targan, B. G. Feagan, S. Vermeire, *et al.* A randomized, double-blind, placebo-controlled study to evaluate the safety, tolerability, and efficacy of AMG 827 in subjects with moderate to severe Crohn's disease. *Gastroenterology* 2012;**143;**E26.

54 W. Reinisch, W. de Villiers, L. Bene, *et al.* Fontolizumab in moderate to severe Crohn's disease: a phase 2, randomized, double-blind, placebo-controlled, multiple-dose study. *Inflamm Bowel Dis* 2010;**16**:233–242.

55 S. Schreiber, R. N. Fedorak, O. H. Nielsan, *et al.* Safety and efficacy of recombinant human interleukin 10 in chronic active Crohn's disease. *Gastroenterology* 2000;**119**:1461–1472.

56 F. E. Buruiana, I. Solà, P. Alonso-Coello. Recombinant human interleukin 10 for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2010;(11):CD005109.

57 B. E. Sands, S. Bank, C. A. Sninsky, *et al.* Preliminary evaluation of safety and activity of recombinant human interleukin 11 in patients with active Crohn's disease. *Gastroenterology* 1999;**117**:58–64.

58 K. R. Herrlinger, T. Witthoeft, A. Raedler, *et al.* Randomized, double blind controlled trial of subcutaneous recombinant human interleukin-11 versus prednisolone in active Crohn's disease. *Am J Gastroenterol* 2006;**101**:793–797.

59 A. E. Slonim, L. Bulone, M. B. Damore, *et al.* A preliminary study of growth hormone therapy for Crohn's disease. *N Engl J Med* 2000;**342**:1633–1637.

60 J. Bassaganya-Riera, R. Hontecillas, W. Horne, *et al.* Conjugated linoleic acid modulates immune responses in patients with mild to moderately active Crohn's disease. *Clin Nutr* 2012;**31**:721–727.

61 J. P. Smith, S. I. Bingaman, F. Ruggiero, *et al.* Therapy with the opioid antagonist naltrexone promotes mucosal healing in active Crohn's disease: a randomized placebo-controlled trial. *Dig Dis Sci* 2011;**56**:2088–2097.

62 R. W. Summers, D. E. Elliott, J. F. Urban Jr, *et al. Trichuris suis* therapy in Crohn's disease. *Gut* 2005;**54**:87–90.

63 J. Croese, J. O'Neil, J. Masson, *et al.* A proof of concept study establishing *Necator americanus* in Crohn's patients and reservoir donors. *Gut* 2006;**55**:136–137.

64 M. T. Abreu, C. Von Tirpitz, P. J. Mannon, *et al.* Extracorporeal photoimmune therapy for refractory moderately active Crohn's disease: a multicenter, open-label study. *Gastroenterology* 2007;**132**:A157.

65 J. R. Korzenik, B. K. Dieckgraefe, J. F. Valentine, *et al.* Sargramostim for active Crohn's disease. *N Engl J Med* 2005;**352**:2193–2201.

66 L. Roth, J. K. Macdonald, J. W. McDonald, N. Chande. Sargramostim (GM-CSF) for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2011;(11):CD008538.

67 A. D. Butterworth, A. G. Thomas, A.K. Akobeng. Probiotics for induction of remission in Crohn's disease. *Cochrane Database Syst Rev* 2008;(3):CD006634.

68 V. E. Rolfe, P. J. Fortun, C. J. Hawkey, F. Bath-Hextall. Probiotics for maintenance of remission in Crohn's disease. *Cochrane Database Syst Rev* 2006;(4):CD004826.

69 K. Whelan, E. M. Quigley. Probiotics in the management of irritable bowel syndrome and inflammatory bowel disease. *Curr Opin Gastroenterol* 2013;**29**:184–189.

70 A. Bourreille, G. Cadiot, G. Le Dreau, *et al. Saccharomyces boulardii* does not prevent relapse of Crohn's disease. *Clin Gastroenterol Hepatol* 2013;**11**:982–987.

71 J. L. Benjamin, C. R. Hedin, A. Koutsoumpas, *et al.* Randomised, double-blind, placebo-controlled trial of fructo-oligosaccharides in active Crohn's disease. *Gut* 2011;**60**:923–929.

72 E. Harrison, P. Allan, A. Ramu, *et al.* Management of intestinal failure in inflammatory bowel disease: small intestinal transplantation or home parenteral nutrition? *World J Gastroenterol* 2014;**20**:3153–3163.

73 N. Sustento-Reodica, P. Ruiz, A. Rogers, *et al.* Recurrent Crohn's disease in transplanted bowel. *Lancet* 1997;**349**:688–691.

74 B. Kaila, D. Grant, N. Pettigrew, *et al.* Crohn's disease recurrence in a small bowel transplant. *Am J Gastroenterol* 2004;**99**:158–162.

75 K. M. Abu-Elmagd, B. Kosmach-Park, G. Costa, *et al.* Long-term survival, nutritional autonomy, and quality of life after intestinal and multivisceral transplantation. *Ann Surg* 2012;**256**:494–508.

76 P. E. Drakos, A. Nagler, R. Or. Case of Crohn's disease in bone marrow transplantation. *Am J Hematol* 1993;**43**:157–158.

77 O. García-Bosch, E. Ricart, J. Panés. Review article: stem cell therapies for inflammatory bowel disease – efficacy and safety. *Aliment Pharmacol Ther* 2010;**32**:939–952.

78 A. Gratwohl, J. Passweg, C. Bocelli-Tyndall, *et al.* Autologous hematopoietic stem cell transplantation for autoimmune diseases. *Bone Marrow Transplant* 2005;**35**:869–879.

79 R. K. Burt, R. M. Craig, F. Milanetti, *et al.* Autologous nonmyeloablative hematopoietic stem cell transplantation in patients with severe anti-TNF refractory Crohn disease: long-term follow-up. *Blood* 2010;**116**:6123–6132.

80 C.J. Hawkey. Stem cells as treatment in inflammatory bowel disease. *Dig Dis* 2012;**30**(Suppl 3):134–139.

81 A. Dignass, J. O. Lindsay, A. Sturm, *et al.* Second European evidence-based consensus on the diagnosis and management of ulcerative colitis part 2: current management. *J Crohns Colitis* 2012;**6**:991–1030.

82 B. G. Feagan, G. R. Greenberg, G. Wild, *et al.* Treatment of ulcerative colitis with a humanized antibody to the $\alpha_4\beta_7$ integrin. *N Engl J Med* 2005;**352**:2499–2507.

83 P. J. Rutgeerts, R. N. Fedorak, D. W. Hommes, *et al.* A randomised phase I study of etrolizumab (rhuMAb β7) in moderate to severe ulcerative colitis. *Gut* 2013;**62**:1122–1130.

84 S. Vermeire, S. O'Byrne, M. Keir, *et al.* Etrolizumab as induction therapy for ulcerative colitis: a randomised, controlled, phase 2 trial. *Lancet* 2014;**384**:309–318.

85 S. Vermeire, S. Ghosh, J. Panes, *et al.* The mucosal addressin cell adhesion molecule antibody PF-00547,659 in ulcerative colitis: a randomised study. *Gut* 2011;**60**:1068–1075.

86 P. B. Miner Jr, R. S. Geary, J. Matson, *et al.* Bioavailability and therapeutic activity of alicaforsen (ISIS 2302) administered as a rectal retention enema to subjects with active ulcerative colitis. *Aliment Pharmacol Ther* 2006;**23**:1427–1434.

87 P. B. Miner Jr, M. K. Wedel, S. Xia, B. F. Baker. Safety and efficacy of two dose formulations of alicaforsen enema compared with mesalazine enema for treatment of mild to moderate left-sided ulcerative colitis: a randomized, double-blind, active-controlled trial. *Aliment Pharmacol Ther* 2006;**23**:1403–1413.

88 S. J. van Deventer, J. A. Tami, M. K. Wedel. A randomised, controlled, double blind, escalating dose study of alicaforsen enema in active ulcerative colitis. *Gut* 2004;**53**:1646–1651.

89 W. J. Sandborn, S. Ghosh, J. Panes, *et al.* Tofacitinib, an oral Janus kinase inhibitor, in active ulcerative colitis. *N Engl J Med* 2012;**367**:616–624.

90 R. A. Kozarek, D. J. Patterson, M. D. Gelfand, *et al.* Methotrexate induces clinical and histologic remission in patients with refractory inflammatory bowel disease. *Ann Intern Med* 1989;**110**:353–356.

91 T. H. Baron, C. D. Truss, C. O. Elson. Low-dose oral methotrexate in refractory inflammatory bowel disease. *Dig Dis Sci* 1993;**38**: 1851–1856.

92 J. R. Cummings, K. R. Herrlinger, S. P. Travis, *et al.* Oral methotrexate in ulcerative colitis. *Aliment Pharmacol Ther* 2005;**21**:385–389.

93 M. Aloi, G. Di Nardo, F. Conte, *et al.* Methotrexate in paediatric ulcerative colitis: a retrospective survey at a single tertiary referral centre. *Aliment Pharmacol Ther* 2010;**32**:1017–1022.

94 S. Willot, A. Noble, C. Deslandres. Methotrexate in the treatment of inflammatory bowel disease: an 8-year retrospective study in a Canadian pediatric IBD center. *Inflamm Bowel Dis* 2011;**17**: 2521–2516.

95 J. L. Anderson, R. J. Edney, K. Whelan. Systematic review: faecal microbiota transplantation in the management of inflammatory bowel disease. *Aliment Pharmacol Ther* 2012;**36**:503–516.

96 T. J. Borody, E. F. Warren, S. Leis, *et al.* Treatment of ulcerative colitis using fecal bacteriotherapy. *J Clin Gastroenterol* 2003;**37**: 42–47.

97 G. Van Assche, I. Dalle, M. Noman, *et al.* A pilot study on the use of the humanized anti-interleukin-2 receptor antibody daclizumab in active ulcerative colitis. *Am J Gastroenterol* 2003;**98**: 369–376.

98 G. Van Assche, W. J. Sandborn, B. G. Feagan, *et al.* Daclizumab, a humanised monoclonal antibody to the interleukin 2 receptor (CD25), for the treatment of moderately to severely active ulcerative colitis: a randomised, double blind, placebo controlled, dose ranging trial. *Gut* 2006;**55**:1568–1574.

99 T. J. Creed, M. R. Norman, C. S. Probert, *et al.* Basiliximab (anti-CD25) in combination with steroids may be an effective new treatment for steroid-resistant ulcerative colitis. *Aliment Pharmacol Ther* 2003;**18**:65–75.

100 T. J. Creed, C. S. Probert, M. N. Norman, *et al.* Basiliximab for the treatment of steroid-resistant ulcerative colitis: further experience in moderate and severe disease. *Aliment Pharmacol Ther* 2006;**23**:1435–1442.

101 B. E. Sands, W. J. Sandborn, T. J. Creed, *et al.* Basiliximab does not increase efficacy of corticosteroids in patients with steroid-refractory ulcerative colitis. *Gastroenterology* 2012;**143**:356–364.

102 S. Plevy, B. Salzberg, G. Van Assche, *et al.* A phase I study of visilizumab, a humanized anti-CD3 monoclonal antibody, in severe steroid-refractory ulcerative colitis. *Gastroenterology* 2007;**133**: 1414–1422.

103 M. Sparrow. Visilizumab in severe ulcerative colitis: good vision but blurry results. *Inflamm Bowel Dis* 2008;**14**:1022–1023.

104 W. J. Sandborn, J. F. Colombel, M. Frankel, *et al.* Anti-CD3 antibody visilizumab is not effective in patients with intravenous corticosteroid-refractory ulcerative colitis. *Gut* 2010;**59**:1485–1492.

105 K. Leiper, K. Martin, A. Ellis, *et al.* Randomised placebo-controlled trial of rituximab (anti-CD20) in active ulcerative colitis. *Gut* 2011;**60**:1520–1526.

106 D. El Fassi, C. H. Nielsen, J. Kjeldsen, *et al.* Ulcerative colitis following B lymphocyte depletion with rituximab in a patient with Graves' disease. *Gut* 2008;**57**:714–715.

107 M. Goetz, R. Atreya, M. Ghalibafian, *et al.* Exacerbation of ulcerative colitis after rituximab salvage therapy. *Inflamm Bowel Dis* 2007;**13**:1365–1368.

108 K. Le Blanc. Mesenchymal stromal cells: tissue repair and immune modulation. *Cytotherapy* 2006;**8**:559–561.

109 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

110 J. W. Kim, S.Y. Kim, S. Y. Park, *et al.* Mesenchymal progenitor cells in the human umbilical cord. *Ann Hematol* 2004;**83**:733–738.

111 A. Erices, P. Conget, J. J. Minguell. Mesenchymal progenitor cells in human umbilical cord blood. *Br J Haematol* 2000;**109**: 235–242.

112 Y. Zhang, C. Li, X. Jiang, *et al.* Human placenta-derived mesenchymal progenitor cells support culture expansion of long-term culture-initiating cells from cord blood $CD34^+$ cells. *Exp Hematol* 2004;**32**:657–664.

113 P. A. Zuk, M. Zhu, P. Ashjian, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**:4279–4295.

114 Q. Zhang, S. Shi, Y. Liu, *et al.* Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis. *J Immunol* 2009;**183**:7787–7798.

115 J. G. Toma, M. Akhavan, K. J. Fernandes, *et al.* Isolation of multipotent adult stem cells from the dermis of mammalian skin. *Nat Cell Biol* 2001;**3**:778–784.

116 C. G. Fan, F. W. Tang, Q. J. Zhang, *et al.* Characterization and neural differentiation of fetal lung mesenchymal stem cells. *Cell Transplant* 2005;**14**:311–321.

117 C. Campagnoli, I. A. Roberts, S. Kumar, *et al.* Identification of mesenchymal stem/progenitor cells in human first-trimester fetal blood, liver, and bone marrow. *Blood* 2001;**98**: 2396–2402.

118 M. J. Hoogduijn, M. J. Crop, A. M. Peeters, *et al.* Human heart, spleen, and perirenal fat-derived mesenchymal stem cells have immunomodulatory capacities. *Stem Cells Dev* 2007;**16**:597–604.

119 H. E. Young, M. L. Mancini, R. P. Wright, *et al.* Mesenchymal stem cells reside within the connective tissues of many organs. *Dev Dyn* 1995;**202**:137–144.

120 P. Alex, N. C. Zachos, T. Nguyen, *et al.* Distinct cytokine patterns identified from multiplex profiles of murine DSS and TNBS-induced colitis. *Inflamm Bowel Dis* 2009;**15**:341–352.

121 K. Le Blanc, L. Tammik, B. Sundberg, *et al.* Mesenchymal stem cells inhibit and stimulate mixed lymphocyte cultures and mitogenic responses independently of the major histocompatibility complex. *Scand J Immunol* 2003;**57**:11–20.

122 A. Bartholomew, C. Sturgeon, M. Siatskas, *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;**30**:42–48.

123 M. Di Nicola, C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**: 3838–3843.

124 M. Krampera, S. Glennie, J. Dyson, *et al.* Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. *Blood* 2003;**101**:3722–3729.

125 I. Prigione, F. Benvenuto, P. Bocca, *et al.* Reciprocal interactions between human mesenchymal stem cells and γδ T cells or invariant natural killer T cells. *Stem Cells* 2009;**27**:693–702.

126 S. Glennie, I. Soeiro, P. J. Dyson, *et al.* Bone marrow mesenchymal stem cells induce division arrest anergy of activated T cells. *Blood* 2005;**105**:2821–2827.

127 F. Benvenuto, S. Ferrari, E. Gerdoni, *et al.* Human mesenchymal stem cells promote survival of T cells in a quiescent state. *Stem Cells* 2007;**25**:1753–1760.

128 K. Akiyama, C. Chen, D. Wang, *et al.* Mesenchymal-stem-cell-induced immunoregulation involves FAS-ligand-/FAS-mediated T cell apoptosis. *Cell Stem Cell* 2012;**10**:544–555.

129 I. Monteleone, G. Monteleone, D. Fina, *et al.* A functional role of flip in conferring resistance of Crohn's disease lamina propria lymphocytes to FAS-mediated apoptosis. *Gastroenterology* 2006;**130**: 389–397.

130 X. J. Liu, J. F. Zhang, B. Sun, *et al.* Reciprocal effect of mesenchymal stem cell on experimental autoimmune encephalomyelitis is mediated by transforming growth factor-β and interleukin-6. *Clin Exp Immunol* 2009;**158**:37–44.

131 M. Di Ianni, B. Del Papa, M. De Ioanni, *et al.* Mesenchymal cells recruit and regulate T regulatory cells. *Exp Hematol* 2008;**36**: 309–318.

132 C. Prevosto, M. Zancolli, P. Canevali, *et al.* Generation of $CD4^+$ or $CD8^+$ regulatory T cells upon mesenchymal stem cell-lymphocyte interaction. *Haematologica* 2007;**92**:881–888.

133 Z. Ye, Y. Wang, H.Y. Xie, S. S. Zheng. Immunosuppressive effects of rat mesenchymal stem cells: involvement of $CD4^+CD25^+$ regulatory T cells. *Hepatobiliary Pancreat Dis Int* 2008;**7**:608–614.

134 E. Zappia, S. Casazza, E. Pedemonte *et al.* Mesenchymal stem cells ameliorate experimental autoimmune encephalomyelitis inducing T cell anergy. *Blood* 2005;**106**:1755–1761.

135 M. A. Gonzalez, E. Gonzalez-Rey, L. Rico, *et al.* Treatment of experimental arthritis by inducing immune tolerance with human adipose-derived mesenchymal stem cells. *Arthritis Rheum* 2009;**60**:1006–1019.

136 A. M. Madec, R. Mallone, G. Afonso, *et al.* Mesenchymal stem cells protect NOD mice from diabetes by inducing regulatory T cells. *Diabetologia* 2009;**52**:1391–1399.

137 K. English, J. M. Ryan, L. Tobin, *et al.* Cell contact, prostaglandin $E_2$ and transforming growth factor beta 1 play non-redundant roles in

human mesenchymal stem cell induction of $CD4^+CD25^{High}$ forkhead box $P3^+$ regulatory T cells. *Clin Exp Immunol* 2009;**156**:149–160.

138 S. A. Patel, J. R. Meyer, S. J. Greco, *et al.* Mesenchymal stem cells protect breast cancer cells through regulatory T cells: role of mesenchymal stem cell-derived TGF-β. *J Immunol* 2010;**184**:5885–5894.

139 K. Nemeth, A. Keane-Myers, J. M. Brown, *et al.* Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci U S A* 2010;**107**:5652–5657.

140 Z. Selmani, A. Naji, I. Zidi, *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce $CD4^+CD25^{high}FOXP3^+$ regulatory T cells. *Stem Cells* 2008;**26**:212–222.

141 N. Rouas-Freiss, A. Naji, A. Durrbach, E. D. Carosella. Tolerogenic functions of human leukocyte antigen G: from pregnancy to organ and cell transplantation. *Transplantation* 2007;**84**:S21–S25.

142 P. Batten, P. Sarathchandra, J. W. Antoniw, *et al.* Human mesenchymal stem cells induce T cell anergy and downregulate T cell alloresponses via the TH2 pathway: relevance to tissue engineering human heart valves. *Tissue Eng* 2006;**12**:2263–2273.

143 Y. S. Choi, J. A. Jeong, D. S. Lim. Mesenchymal stem cell-mediated immature dendritic cells induce regulatory T cell-based immunosuppressive effect. *Immunol Invest* 2012;**41**:214–229.

144 J. Maggini, G. Mirkin, I. Bognanni, *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE* 2010;**5**:e9252.

145 E. Gonzalez-Rey, P. Anderson, M. A. González, *et al.* Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut* 2009;**58**:929–939.

146 M. A. González, E. Gonzalez-Rey, L. Rico, *et al.* Adipose-derived mesenchymal stem cells alleviate experimental colitis by inhibiting inflammatory and autoimmune responses. *Gastroenterology* 2009;**136**:978–989.

147 L. Liang, C. Dong, X. Chen, *et al.* Human umbilical cord mesenchymal stem cells ameliorate mice trinitrobenzene sulfonic acid (TNBS)-induced colitis. *Cell Transplant* 2011;**20**:1395–1408.

148 X. W. He, X. S. He, L. Lian, *et al.* Systemic infusion of bone marrow-derived mesenchymal stem cells for treatment of experimental colitis in mice. *Dig Dis Sci* 2012;**57**:3136–3144.

149 B. Parekkadan, R. Upadhyay, J. Dunham, *et al.* Bone marrow stromal cell transplants prevent experimental enterocolitis and require host $CD11b^+$ splenocytes. *Gastroenterology* 2011;**140**:966–975.

150 Q. Q. Chen, L. Yan, C. Z. Wang, *et al.* Mesenchymal stem cells alleviate TNBS-induced colitis by modulating inflammatory and autoimmune responses. *World J Gastroenterol* 2013;**19**:4702–4717.

151 A. Augello, R. Tasso, S. M. Negrini, *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;**35**:1482–1490.

152 W. Deng, Q. Han, L. Liao, *et al.* Effects of allogeneic bone marrow-derived mesenchymal stem cells on T and B lymphocytes from BXSB mice. *DNA Cell Biol* 2005;**24**:458–463.

153 A. Corcione, F. Benvenuto, E. Ferretti, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**:367–372.

154 E. Traggiai, S. Volpi, F. Schena, *et al.* Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008;**26**:562–569.

155 P. A. Sotiropoulou, S. A. Perez, A. D. Gritzapis, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**:74–85.

156 G. M. Spaggiari, A. Capobianco, H. Abdelrazik, *et al.* Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008;**111**:1327–1333.

157 M. Krampera, L. Cosmi, R. Angeli, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**:386–398.

158 I. Rasmusson, O. Ringdén, B. Sundberg, K. Le Blanc. Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. *Transplantation* 2003;**76**:1208–1213.

159 P. A. Sotiropoulou, S. A. Perez, M. Salagianni, *et al.* Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells. *Stem Cells* 2006;**24**:462–471.

160 A. J. Nauta, A. B. Kruisselbrink, E. Lurvink, *et al.* Mesenchymal stem cells inhibit generation and function of both $CD34^+$-derived and monocyte-derived dendritic cells. *J Immunol* 2006;**177**:2080–2087.

161 R. Ramasamy, H. Fazekasova, E. W. Lam, *et al.* Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 2007;**83**:71–76.

162 K. English, F. P. Barry, B. P. Mahon. Murine mesenchymal stem cells suppress dendritic cell migration, maturation and antigen presentation. *Immunol Letters* 2008;**115**:50–58.

163 S. Aggarwal, M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**:1815–1822.

164 S. Chiesa, S. Morbelli, S. Morando, *et al.* Mesenchymal stem cells impair *in vivo* T cell priming by dendritic cells. *Proc Natl Acad Sci U S A* 2011;**108**:17384–17389.

165 Y. P. Li, S. Paczesny, E. Lauret, *et al.* Human mesenchymal stem cells license adult $CD34^+$ hemopoietic progenitor cells to differentiate into regulatory dendritic cells through activation of the Notch pathway. *J Immunol* 2008;**180**:1598–1608.

166 B. Zhang, R. Liu, D. Shi, *et al.* Mesenchymal stem cells induce mature dendritic cells into a novel Jagged-2-dependent regulatory dendritic cell population. *Blood* 2009;**113**:46–57.

167 K. Németh, A. Leelahavanichkul, P. S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**:42–49.

168 P. Anderson, L. Souza-Moreira, M. Morell, *et al.* Adipose-derived mesenchymal stromal cells induce immunomodulatory macrophages which protect from experimental colitis and sepsis. *Gut* 2013;**62**:1131–1141.

169 G. Ren, J. Su, L. Zhang, *et al.* Species variation in the mechanisms of mesenchymal stem cell-mediated immunosuppression. *Stem Cells* 2009;**27**:1954–1962.

170 M. Matysiak, W. Orlowski, M. Fortak-Michalska, *et al.* Immunoregulatory function of bone marrow mesenchymal stem cells in

EAE depends on their differentiation state and secretion of PGE2. *J Neuroimmunol* 2011;**233**:106–111.

171 R. Meisel, A. Zibert, M. Laryea, *et al.* Human bone marrow stromal cells inhibit allogeneic T cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**:4619–4621.

172 K. Sato, K. Ozaki, I. Oh, *et al.* Nitric oxide plays a critical role in suppression of T cell proliferation by mesenchymal stem cells. *Blood* 2007;**109**:228–234.

173 G. Ren, L. Zhang, X. Zhao, *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**:141–150.

174 A. Nasef, N. Mathieu, A. Chapel, *et al.* Immunosuppressive effects of mesenchymal stem cells: involvement of HLA-G. *Transplantation* 2007;**84**:231–237.

175 L. A. Ortiz, M. Dutreil, C. Fattman, *et al.* Interleukin 1 receptor antagonist mediates the antiinflammatory and antifibrotic effect of mesenchymal stem cells during lung injury. *Proc Natl Acad Sci U S A* 2007;**104**:11002–11007.

176 H. Choi, R. H. Lee, N. Bazhanov, *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood* 2011;**118**:330–338.

177 H. S. Kim, T. H. Shin, B. C. Lee, *et al.* Human umbilical cord blood mesenchymal stem cells reduce colitis in mice by activating NOD2 signaling to COX2. *Gastroenterology* 2013;**145**:1392–1403.

178 B. Parekkadan, A. L. Fletcher, M. Li, *et al.* Aire controls mesenchymal stem cell-mediated suppression in chronic colitis. *Mol Ther* 2012;**20**:178–186.

179 O. DelaRosa, E. Lombardo. Modulation of adult mesenchymal stem cells activity by toll-like receptors: implications on therapeutic potential. *Mediat Inflamm* 2010;**2010**:865601.

180 R. S. Waterman, S. L. Tomchuck, S. L. Henkle, A. M. Betancourt. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* 2010;**5**:e10088.

181 M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, *et al.* Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 2007;**109**:1422–1432.

182 M. Duijvestein, M. E. Wildenberg, M. M. Welling, *et al.* Pretreatment with interferon-γ enhances the therapeutic activity of mesenchymal stromal cells in animal models of colitis. *Stem Cells* 2011;**29**:1549–1558.

183 H. Fan, G. Zhao, L. Liu, *et al.* Pre-treatment with IL-1β enhances the efficacy of MSC transplantation in DSS-induced colitis. *Cell Mol Immunol* 2012;**9**:473–481.

184 K. Le Blanc, C. Tammik, K. Rosendahl, *et al.* HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. *Exp Hematol* 2003;**31**:890–896.

185 W. T. Tse, J. D. Pendleton, W. M. Beyer, *et al.* Suppression of allogeneic T cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* 2003;**75**:389–397.

186 G. M. Spaggiari, A. Capobianco, S. Becchetti, *et al.* Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;**107**:1484–1490.

187 M. Francois, R. Romieu-Mourez, S. Stock-Martineau, *et al.* Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. *Blood* 2009;**114**:2632–2638.

188 R. Romieu-Mourez, M. Francois, M. N. Boivin, *et al.* Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-γ, TGF-β, and cell density. *J Immunol* 2007;**179**:1549–1558.

189 W. K. Chan, A. S. Lau, J. C. Li, *et al.* MHC expression kinetics and immunogenicity of mesenchymal stromal cells after short-term IFN-γ challenge. *Exp Hematol* 2008;**36**:1545–1555.

190 J. Stagg, S. Pommey, N. Eliopoulos, *et al.* Interferon-γ-stimulated marrow stromal cells: a new type of nonhematopoietic antigen-presenting cell. *Blood* 2006;**107**:2570–2577.

191 A. J. Nauta, G. Westerhuis, A. B. Kruisselbrink, *et al.* Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006;**108**:2114–2120.

192 P. Sbano, A. Cuccia, B. Mazzanti, *et al.* Use of donor bone marrow mesenchymal stem cells for treatment of skin allograft rejection in a preclinical rat model. *Arch Dermatol Res* 2008;**300**:115–124.

193 S. Schu, M. Nosov, L. O'Flynn, *et al.* Immunogenicity of allogeneic mesenchymal stem cells. *J Cell Mol Med* 2012;**16**:2094–2103.

194 X-P. Huang, Z. Sun, Y. Miyagi, *et al.* Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation* 2010;**122**:2419–2429.

195 S. M. Devine, C. Cobbs, M. Jennings, *et al.* Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003;**101**:2999–3001.

196 F. Tanaka, K. Tominaga, M. Ochi, *et al.* Exogenous administration of mesenchymal stem cells ameliorates dextran sulfate sodium-induced colitis via anti-inflammatory action in damaged tissue in rats. *Life Sci* 2008;**83**:771–779.

197 M. T. Castelo-Branco, I. D. Soares, D. V. Lopes, *et al.* Intraperitoneal but not intravenous cryopreserved mesenchymal stromal cells home to the inflamed colon and ameliorate experimental colitis. *PLoS ONE* 2012;**7**:e33360.

198 E. Spaeth, A. Klopp, J. Dembinski, *et al.* Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Ther* 2008;**15**:730–738.

199 H. Yagi, A. Soto-Gutierrez, B. Parekkadan, *et al.* Mesenchymal stem cells: Mechanisms of immunomodulation and homing. *Cell Transplant* 2010;**19**:667–679.

200 I. K. Ko, B. G. Kim, A. Awadallah, *et al.* Targeting improves MSC treatment of inflammatory bowel disease. *Mol Ther* 2010;**18**:1365–1372.

201 S. Wakitani, T. Saito, A. I. Caplan. Myogenic cells derived from rat bone marrow mesenchymal stem cells exposed to 5-azacytidine. *Muscle Nerve* 1995;**18**:1417–1426.

202 R. A. Rose, A. Keating, P. H. Backx. Do mesenchymal stromal cells transdifferentiate into functional cardiomyocytes? *Circ Res* 2008;**103**:e120.

203 O. K. Lee, T. K. Kuo, W. M. Chen, *et al.* Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;**103**:1669–1675.

204 D. Woodbury, E. J. Schwarz, D. J. Prockop, I. B. Black. Adult rat and human bone marrow stromal cells differentiate into neurons. *J Neurosci Res* 2000;**61**:364–370.

205 P. Tropel, N. Platet, J. C. Platel, *et al.* Functional neuronal differentiation of bone marrow-derived mesenchymal stem cells. *Stem Cells* 2006;**24**:2868–2876.

206 K. Singaravelu, B. J. Padanilam. *In vitro* differentiation of MSC into cells with a renal tubular epithelial-like phenotype. *Ren Fail* 2009;**31**:492–502.

207 R. E. Schwartz, M. Reyes, L. Koodie, *et al.* Multipotent adult progenitor cells from bone marrow differentiate into functional hepatocyte-like cells. *J Clin Invest* 2002;**109**:1291–1302.

208 R. Taléns-Visconti, A. Bonora, R. Jover, *et al.* Human mesenchymal stem cells from adipose tissue: differentiation into hepatic lineage. *Toxicol In Vitro* 2007;**21**:324–329.

209 M. J. Seo, S. Y. Suh, Y. C. Bae, J. S. Jung Differentiation of human adipose stromal cells into hepatic lineage *in vitro* and *in vivo*. *Biochem Biophys Res Commun* 2005;**328**:258–264.

210 V. Sordi. Mesenchymal stem cell homing capacity. *Transplantation* 2009;**87**:S42–S45.

211 T. J. Cashman, V. Gouon-Evans, K. D. Costa. Mesenchymal stem cells for cardiac therapy: practical challenges and potential mechanisms. *Stem Cell Rev* 2013;**9**:254–265.

212 U. Kunter, S. Rong, Z. Djuric, *et al.* Transplanted mesenchymal stem cells accelerate glomerular healing in experimental glomerulonephritis. *J Am Soc Nephrol* 2006;**17**:2202–2212.

213 M. B. Herrera, B. Bussolati, S. Bruno, *et al.* Mesenchymal stem cells contribute to the renal repair of acute tubular epithelial injury. *Int J Mol Med* 2004;**14**:1035–1041.

214 R. P. Meier, Y. D. Müller, P. Morel, *et al.* Transplantation of mesenchymal stem cells for the treatment of liver diseases, is there enough evidence? *Stem Cell Res* 2013;**11**:1348–1364.

215 A. C. Inamdar, A. A. Inamdar. Mesenchymal stem cell therapy in lung disorders: pathogenesis of lung diseases and mechanism of action of mesenchymal stem cell. *Exp Lung Res* 2013;**39**:315–327.

216 F. Xu, Y. Hu, J. Zhou, X. Wang. Mesenchymal stem cells in acute lung injury: are they ready for translational medicine? *J Cell Mol Med* 2013;**17**:927–935.

217 G. Paul, S. V. Anisimov. The secretome of mesenchymal stem cells: potential implications for neuroregeneration. *Biochimie* 2013;**95**:2246–2256.

218 Y. Hayashi, S. Tsuji, M. Tsujii, *et al.* Topical implantation of mesenchymal stem cells has beneficial effects on healing of experimental colitis in rats. *J Pharmacol Exp Ther* 2008;**326**:523–531.

219 T. Yabana, Y. Arimura, H. Tanaka, *et al.* Enhancing epithelial engraftment of rat mesenchymal stem cells restores epithelial barrier integrity. *J Pathol* 2009;**218**:350–359.

220 D. J. Prockop. "Stemness" does not explain the repair of many tissues by mesenchymal stem/multipotent stromal cells (MSCs). *Clin Pharmacol Ther* 2007;**82**:241–243.

221 S. Kachgal, A. J. Putnam. Mesenchymal stem cells from adipose and bone marrow promote angiogenesis via distinct cytokine and protease expression mechanisms. *Angiogenesis* 2011;**14**:47–59.

222 A. I. Caplan. Why are MSCs therapeutic? New data: new insight. *J Pathol* 2009;**217**:318–324.

223 D. García-Olmo, M. García-Arranz, D. Herreros, *et al.* A phase I clinical trial of the treatment of Crohn's fistula by adipose mesenchymal stem cell transplantation. *Dis Colon Rectum* 2005;**48**:1416–1423.

224 D. García-Olmo, M. García-Arranz, D. Herreros. Expanded adipose-derived stem cells for the treatment of complex perianal fistula including Crohn's disease. *Expert Opin Biol Ther* 2008;**8**:1417–1423.

225 D. García-Olmo, D. Herreros, I. Pascual, *et al.* Expanded adipose-derived stem cells for the treatment of complex perianal fistula: a phase II clinical trial. *Dis Colon Rectum* 2009;**52**:79–86.

226 H. Guadalajara, D. Herreros, P. De-La-Quintana, *et al.* Long-term follow-up of patients undergoing adipose-derived adult stem cell administration to treat complex perianal fistulas. *Int J Colorectal Dis* 2012;**27**:595–600.

227 Y. B. Cho, W. Y. Lee, K. J. Park, *et al.* Autologous adipose tissue-derived stem cells for the treatment of Crohn's fistula: a phase I clinical study. *Cell Transplant* 2013;**22**:279–285.

228 W. Y. Lee, K. J. Park, Y. B. Cho, *et al.* Autologous adipose tissue-derived stem cells treatment demonstrated favorable and sustainable therapeutic effect for Crohn's fistula. *Stem Cells* 2013;**31**:2575–2581.

229 R. Ciccocioppo, M. E. Bernardo, A. Sgarella, *et al.* Autologous bone marrow-derived mesenchymal stromal cells in the treatment of fistulising Crohn's disease. *Gut* 2011;**60**:788–798.

230 F. de la Portilla, F. Alba, D. García-Olmo, *et al.* Expanded allogeneic adipose-derived stem cells (eASCs) for the treatment of complex perianal fistula in Crohn's disease: results from a multicenter phase I/IIa clinical trial. *Int J Colorectal Dis* 2013;**28**:313–323.

231 M. Duijvestein, A. C. Vos, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut* 2010;**59**:1662–1669.

232 J. Onken, D. Gallup, J. Hanson, *et al.* Successful outpatient treatment of refractory Crohn's disease using adult mesenchymal stem cells. In: *Proceedings of the American College of Gastroenterology Conference 2006*, Las Vegas, NV, Abstract 121.

233 J. Liang, H. Zhang, D. Wang, *et al.* Allogeneic mesenchymal stem cell transplantation in seven patients with refractory inflammatory bowel disease. *Gut* 2012;**61**:468–469.

234 Bonab MM, Alimoghaddam K, Talebian F, *et al.* Aging of mesenchymal stem cell *in vitro*. *BMC Cell Biol* 2006;**7**:14.

235 D. García-Olmo, D. Herreros, M. Pascual, *et al.* Treatment of enterocutaneous fistula in Crohn's disease with adipose-derived stem cells: a comparison of protocols with and without cell expansion. *Int J Colorectal Dis* 2009;**24**:27–30.

236 I. Bochev, G. Elmadjian, D. Kyurkchiev, *et al.* Mesenchymal stem cells from human bone marrow or adipose tissue differently modulate mitogen-stimulated B-cell immunoglobulin production *in vitro*. *Cell Biol Int* 2008;**32**:384–393.

237 E. Ivanova-Todorova, I. Bochev, M. Mourdjeva, *et al.* Adipose tissue-derived mesenchymal stem cells are more potent suppressors of dendritic cells differentiation compared to bone marrow-derived mesenchymal stem cells. *Immunol Lett* 2009;**126**:37–42.

238 L. Y. Sun, H. Y. Zhang, X. B. Feng, *et al.* Abnormality of bone marrow-derived mesenchymal stem cells in patients with systemic lupus erythematosus. *Lupus* 2007;**16**:121–128.

239 C. Bocelli-Tyndall, L. Bracci, G. Spagnoli, *et al.* Bone marrow mesenchymal stromal cells (BM-MSCs) from healthy donors and auto-immune disease patients reduce the proliferation of autologous- and allogeneic-stimulated lymphocytes *in vitro*. *Rheumatology (Oxford)* 2007;**46**:403–408.

240 L. Dinesen, A. Wang, F. Vianello, *et al.* Mesenchymal stem cells administered via novel selective mesenteric artery cannulation for

the treatment of severe refractory Crohn's disease. *J Crohns Colitis* 2010;**3**:S51–S52.

241 M. Duijvestein, I. Molendijk, H. Roelofs, *et al.* Mesenchymal stromal cell function is not affected by drugs used in the treatment of inflammatory bowel disease. *Cytotherapy* 2011;**13**:1066–1073.

242 F. Baron, R. Storb. Mesenchymal stromal cells: a new tool against graft-versus-host disease? *Biol Blood Marrow Transplant* 2012;**18**: 822–840.

243 K. Le Blanc, F. Frassoni, L. Ball, *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**:1579–1586.

244 F. Baron, C. Lechanteur, E. Willems, *et al.* Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-mismatched allogeneic transplantation following nonmyeloablative conditioning. *Biol Blood Marrow Transplant* 2010;**16**:838–847.

245 J Tan, W. Wu, X. Xu, *et al.* Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* 2012;**307**:1169–1177.

246 S. Wakitani, T. Okabe, S. Horibe, *et al.* Safety of autologous bone marrow-derived mesenchymal stem cell transplantation for cartilage repair in 41 patients with 45 joints followed for up to 11 years and 5 months. *J Tissue Eng Regen Med* 2011;**5**:146–150.

247 W. R. Otto, N. A. Wright. Mesenchymal stem cells: from experiment to clinic. *Fibrogenesis Tissue Repair* 2011;**4**:20.

248 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**:e47559.

249 Y. F. Zhou, M. Bosch-Marce, H. Okuyama, *et al.* Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. *Cancer Res* 2006;**66**:10849–10854.

250 M. Miura, Y. Miura, H. M. Padilla-Nash, *et al.* Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells* 2006;**24**:1095–1103.

251 F. Casiraghi, G. Remuzzi, M. Abbate, N. Perico. Multipotent mesenchymal stromal cell therapy and risk of malignancies. *Stem Cell Rev* 2013;**9**:65–79.

252 M. E. Bernardo, N. Zaffaroni, F. Novara, *et al.* Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 2007;**67**: 9142–9149.

253 D.J. Prockop, M. Brenner, W. E. Fibbe, *et al.* Defining the risks of mesenchymal stromal cell therapy. *Cytotherapy* 2010;**12**:576–578.

254 Z. Chen, X. He, X. He, *et al.* Bone marrow mesenchymal stem cells ameliorate colitis-associated tumorigenesis in mice. *Biochem Biophys Res Commun* 2014;**450**:1402–1408.

255 G. Ren, X. Zhao, Y. Wang, *et al.* CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα. *Cell Stem Cell* 2012;**11**:812–824.

256 B. Song, B. Kim, S. H. Choi, *et al.* Mesenchymal stromal cells promote tumor progression in fibrosarcoma and gastric cancer cells. *Korean J Pathol* 2014;**48**:217–224.

257 H. Lee Ryang, N. Yoon, J. C. Reneau, D. J. Prockop. Preactivation of human MSCs with TNF-a enhances tumor-suppressive activity. *Cell Stem Cell* 2012;**11**:825–835.

258 S. H. Abd-Allah, S. M. Shalaby, A. S. El-Shal, *et al.* Effect of bone marrow-derived mesenchymal stromal cells on hepatoma. *Cytotherapy* 2014;**16**:1197–1206.

259 C. Yang, D. Lei, W. Ouyang, *et al.* Conditioned media from human adipose tissue-derived mesenchymal stem cells and umbilical cord-derived mesenchymal stem cells efficiently induced the apoptosis and differentiation in human glioma cell lines *in vitro*. *Biomed Res Int* 2014;**2014**:109389.

260 A. Mantovani. MSCs, macrophages, and cancer: a dangerous ménage-à-trois. *Cell Stem Cell* 2012;**11**:730–732.

261 G. Lazennec, C. Jorgensen. Concise review: adult multipotent stromal cells and cancer: risk or benefit? *Stem Cells* 2008;**26**: 1387–1394.

262 H. Ning, F. Yang, M. Jiang, *et al.* The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* 2008;**22**:593–539.

CHAPTER 51

# Mesenchymal stromal cells targeting kidney disease: benefits of a combined therapeutic approach

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## 51.1 Introduction

Mesenchymal stromal cells (MSCs) show great promise in ameliorating kidney disease. These multipotent fibroblast-like stromal cells can migrate to damaged kidneys, where they selectively home to inflammatory cues and alter the course of injury. MSCs can elicit endogenous repair through paracrine and/or endocrine mechanisms that modulate the immune response, ultimately allowing for cellular replacement. This chapter will discuss the features of MSCs and evidence for their homing and engraftment to sites of inflammation as well as their unique immunomodulatory properties and the factors they release that protect against kidney injury. New approaches using MSCs as delivery vehicles for secreted proteins and methods for improving efficacy will also be reviewed. Despite uncertainty over mechanisms mediating protection, MSCs are currently in clinical trials for a range of acute and chronic diseases, including kidney disorders. This chapter will discuss challenges in applying MSCs in a setting of chronic kidney disease (CKD). The potential of a combined therapeutic approach using MSCs and an antifibrotic, relaxin, to aid in MSC viability and the promotion of endogenous repair, cellular replacement, and attenuation of fibrosis will be highlighted.

## 51.2 Immune modulation and protective effects of mesenchymal stromal cells

Nonrenal stromal cells such as mesenchymal stromal cells (MSCs) have been identified as a potential therapy for kidney disease owing to their immunomodulatory properties and their ability to ameliorate acute kidney damage through endocrine and paracrine mechanisms [1]. MSCs have been characterized in a wide variety of fetal tissues during early gestation. However, by the third trimester of pregnancy MSC numbers rapidly decline in the periphery and eventually establish themselves in the bone marrow (BM) [2]. Postnatally, MSCs exist in virtually all organs, including the kidney. However, their immunophenotype and differentiation potential differ from MSCs isolated from other sources, such as the BM [3].

In the setting of experimental kidney disease, MSCs derived from the BM have been extensively studied. These cells are classified according to the expression of surface markers, including CD73, CD90, and CD105, and the lack of hematopoietic markers, adherence to plastic in culture with a fibroblast-like appearance, and the capacity to differentiate into linages of the mesoderm, such as bone, fat, and cartilage under appropriate culture conditions [4]. BM-MSCs are an attractive cell source for

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therapeutic delivery as they are relatively easily obtained from BM, readily cultured into adequate numbers for administration, and have demonstrated immunomodulatory properties [5].

While the exact mechanisms of MSC-induced renal repair are yet to be fully elucidated, it has been established that, rather than direct transdifferentiation into kidney cells, MSCs exhibit unique immunomodulatory effects on both the innate and adaptive immune systems, while releasing many growth factors to elicit tissue repair [6,7] (Figure 51.1).

MSC administration in experimental settings of kidney disease results in the preservation of kidney architecture and an increase in kidney function (as measured by glomerular filtration rate (GFR)), and coinciding with an increase in tubular epithelial cell proliferation and decreased cell apoptosis [8]. (Kidney function impairment is indicated by a GFR value below the normal range, a serum creatinine value above the normal range, and a urea or blood urea nitrogen level above the normal range.) When cytokine and growth factor expression were analyzed from whole

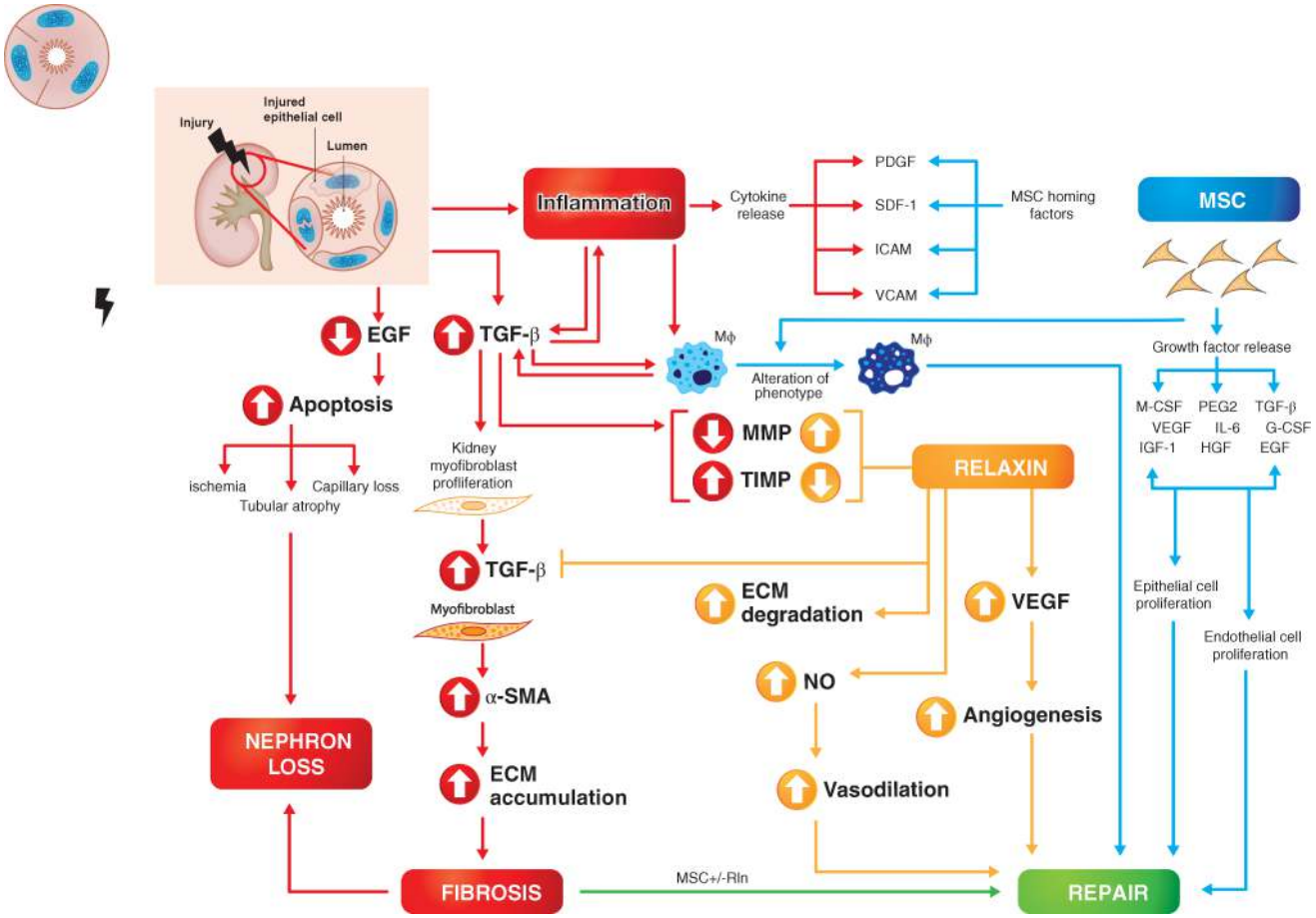



**Figure 51.1** The mechanisms of MSC-mediated repair after kidney injury. Diagrammatic representation of the progression of renal fibrosis and nephron loss, and the regulatory pathways targeted by MSCs and relaxin that promote structural and functional kidney repair. Following injury, tubular epithelial cells release a range of chemokines that alter the local kidney microenvironment. Epidermal growth factor (EGF) expression is decreased, causing apoptosis of endothelial and epithelial cells, leading to hypoxia and ultimately nephron loss. Transforming growth factor (TGF)-β is also released and serves as an important mediator of fibroblast transformation into myofibroblasts, the main collagen-producing cells in the injured kidney. Activated myofibroblasts express α-smooth muscle actin (α-SMA) and augment the deposition of extracellular matrix (ECM), exacerbating hypoxia and cell death, and ultimately leading to progressive interstitial fibrosis. This is compounded by TGF-β-induced inhibition of matrix metalloproteinases (MMPs) and the upregulation of tissue inhibitors of MMPs (TIMPs). Inflammation stimulates the release of TGF-β and other cytokines, leading to immune cell infiltration. Inflammatory release of cytokines, such as platelet-derived growth factor (PDGF), stromal cell-derived factor (SDF)-1, intracellular adhesion molecule (ICAM), and vascular cell adhesion molecule (VCAM), also serve as chemoattractants for MSC homing. At the site of kidney injury, MSCs alter the microenvironment of the kidney by interacting with macrophages, and by releasing trophic growth factors that result in the proliferation of epithelial and endothelial cells, resolving inflammation and promoting endogenous repair. If fibrosis is already established, the efficacy of MSCs may be reduced. Therefore, altering the local environment with an antifibrotic agent, such as relaxin (Rln), can aid in MSC-mediated repair. Exogenous relaxin (serelaxin) directly inhibits the downstream pathways of TGF-β, preventing fibrosis. Additionally, relaxin promotes MMP activity and downregulates TIMPs, leading to further ECM degradation. Additionally, relaxin promotes angiogenesis and vasodilation through the upregulation of vascular endothelial growth factor (VEGF) and nitric oxide (NO), contributing to kidney remodeling after injury. G-CSF: granulocyte colony-stimulating factor; HGF: hepatocyte growth factor; IGF: insulin-like growth factor; IL: interleukin; M-CSF: macrophage colony-stimulating factor; $PGE_2$: prostaglandin $E_2$. (See color plate section.)

kidneys, it was found that MSC administration resulted in an upregulation of anti-inflammatory cytokines, IL-10, basic fibroblast growth factor (bFGF), and TGF-α, and a decrease in the proinflammatory cytokines IL-1β, tumor necrosis factor (TNF)-α, interferon gamma (IFN-γ), and inducible nitric oxide synthase (iNOS) [8], suggesting that secreted factors released by MSCs influence the microenvironment of the injured kidney and mediate the reparative function. Further evidence for the protective paracrine mechanisms of MSCs in kidney disease emanates from experiments that administered MSC-conditioned media (CM) in a cisplatin model of kidney injury. Mice receiving CM had better survival outcomes, improved histopathology correlating with an increase in GFR, and a decrease in tubular apoptosis compared with vehicle-injected mice [9]. Furthermore, administration of MSCs in a rat remnant model of kidney disease influenced the immune system by downregulating IL-6 and TNF-α while increasing IL-4 and IL-10 expression levels [10]. Additionally, in obstructive nephropathy, expression of the proinflammatory cytokine IFN-γ was significantly decreased and IL-10 was significantly increased in MSC-treated animals when compared with sham- and saline-injected control mice [11].

The anti-inflammatory effects of MSCs may also be due to downstream effects on dendritic cells (DCs), antigen-presenting cells that can trigger either proinflammatory or anti-inflammatory pathways, and are involved in the early innate immune response [7]. MSCs may prevent the maturation of DCs by inhibiting the release of proinflammatory cytokines such as IL-12 and TNF-α, while also increasing secretion of IL-10, suggesting that MSCs can influence DCs and trigger a helper T (Th)2 anti-inflammatory profile [12,13]. Moreover, MSCs release a plethora of cytokines that are responsible for their therapeutic effect, with IL-6 [14] and $PGE_2$ [15] being implicated in preventing DC maturation. $PGE_2$, IL-10, and TGF-β released from MSCs are also seemingly important for the inhibition of natural killer (NK) cell proliferation, cytokine release, and lytic ability. However, this effect is lost if the NK cells had previously been exposed to IL-12 [16]. Of further significance, Spaggiari *et al.* characterized the ligands expressed on MSCs that were able to bind to specific receptors on IL-12-stimulated NK cells and induced lysis of both allogeneic and autologous MSCs [17], which may provide an explanation of the null effect on NK cells derived from human peripheral blood mononuclear cells (PBMCs) [18].

B and T cells are major players in the adaptive immune response, and MSCs may be involved in inhibiting the proliferation, differentiation, and activation of both. *In vitro* culture of MSCs and stimulated B cells caused a decrease in B cell proliferation, maturation, and immunoglobulin M production [19]. MSC-derived soluble factors may induce this effect as MSC-CM inhibited proliferation and immunoglobulin production to the same extent as that of MSCs [20]. Data have suggested that the release of IFN-γ from NK and T cells upregulates indoleamine 2,3-dioxygenase (IDO) expression from MSCs, which is responsible for the suppressive effects of MSCs on activated B, T, and NK cells [21,22]. While contrasting studies demonstrate that MSCs may enhance B cell proliferation and maturation [23], a study by Rosado *et al.* used MSCs and PBMCs from healthy and kidney transplant patients to show that the efficacy of MSC-mediated B cell inhibition was dependent on the presence of T cells and their cell-to-cell contact with MSCs *in vitro* [24]. In order for MSCs to modulate T cells, they first need to be activated by IFN-γ, and/or TNF-α, which may be released from the T cells themselves or the tissue microenvironment [25]. This in turn causes the release a myriad of growth factors and cytokines secreted from MSCs, such as IL-6, IL-10, $PGE_2$, TGF-β, HGF, and IDO [7]. As such, MSCs can inhibit T cell proliferation, cytokine production, and cytotoxicity, an effect that is abrogated by blocking the IFN-γ receptor on MSCs [22].

Of further note, a study performed by Aggarwal and Pittenger [26] suggested that the presence of MSCs in an inflammatory environment modulates inflammation by altering cytokine release from DCs, NK cells, and T cells. More specifically, the presence of MSCs induced naive T cells (Th1) to decrease IFN-γ release and helper T cells (Th2) to increase IL-4 secretion, suggesting a shift from a proinflammatory (Th1) to anti-inflammatory (Th2) microenvironment [26]. $PGE_2$ levels increased in the MSC/immune cell culture supernatant, in addition to vascular endothelial growth factor (VEGF), suggesting that these molecules are released by MSCs and likely involved in modulating inflammation [26]. Interestingly, when MSCs from living kidney donors were cultured with stimulated PBMCs from their organ recipients the proliferation and activation of T cells was significantly hampered and likely mediated by an upregulation of IL-10 and IDO [18]. The fact that MSCs can modulate the immune system potentially makes them an attractive therapy for immune-related diseases, such as graft-versus-host disease occurring after allogeneic hematopoietic stem cell transplantation (see Chapter 57) and in solid organ transplantation (see Chapter 56). Additionally, it is also an important characteristic when considering MSC therapy for chronic diseases where ensuing inflammation is present, such as the progression of CKD.

The direct interaction of MSCs and macrophages has been reported to be critical in the reparative effects of MSCs. While the ablation of macrophages in an ischemia reperfusion (IR) model of renal disease resulted in a significant attenuation of fibrosis [27], macrophages are increasingly being recognized as having a more complex role in tissue repair and remodeling, with both inflammatory and reparative phenotypes observed, dependent on cues within the microenvironment [28–30]. Németh *et al.* demonstrated that MSCs can reprogram macrophages and induce the release of IL-10 in a $PGE_2$-dependent manner inducing organ repair in a sepsis model of injury, yet the ablation of macrophages rendered MSCs ineffective [31]. Macrophage-dependent reparative effects have also been observed by alteration of the microenvironment using colony-stimulating factor (CSF)-1 [32]. Analysis of macrophages obtained from IR injured

kidneys treated with CSF-1 showed that they had an altered gene expression profile and were induced to release IGF-1, compared to macrophages not exposed to CSF-1 [32]. This may be one of the main mechanisms by which CSF-1 induces renal protection [32]. A study from our laboratory demonstrated that direct or indirect co-culture of human MSCs with stimulated murine-derived macrophages upregulated genes associated with an anti-inflammatory-like state [33]. The supernatant of these cultures was screened for human growth factors and cytokines that might have been responsible for this alteration, and EGF, granulocyte-macrophage CSF, chemokine motif C–X–C ligand 1, IL-6, monocyte chemotactic protein, platelet-derived growth factor (PDGF)-AA, and chemokine motif C–C ligand 5 were detected [33].

## 51.3 Mesenchymal stromal cell homing, recruitment, and tracking

Understanding the processes that control MSC homing, migration, and engraftment to injured tissues where they exert their reparative effects is of great importance for the development of more selective MSC therapies. First, the weak interaction between MSCs and P-selectin expressed on the endothelium likely facilitates MSC rolling and tethering along the endothelium toward a stromal-cell-derived growth factor (SDF)-1 and hyaluronic acid (HA) gradient originating from the inflamed tissue [1,34]. Following ischemic kidney injury in mice, Tögel *et al* were the first to demonstrate that surviving tubular epithelial cells upregulate SDF-1 and its receptor, C–X–C motif chemokine receptor 4 (CXCR4) [35]. MSCs also express CXCR4, which is critical for the migration and homing of MSCs toward SDF-1. Delivery of MSCs that were preincubated with an anti-CXCR4 blocking antibody has been reported to significantly reduce the number of MSCs localizing in the ischemic kidney [35]. CD44 is also expressed on MSCs [36], which is the surface receptor for HA, and it has also been suggested that it plays a role in MSC homing. Herrera *et al.* [37] used a model of glycerol-induced kidney injury to confirm that HA expression was increased in response to injury and that $CD44^{+}$ MSCs migrated to, and localized in, the injured kidney. However, MSCs that were obtained from the BM of CD44 knockout mice failed to localize in the damaged kidney. This effect could be reversed if the $CD44^{-/-}$ cells were transfected with wild-type CD44, indicating that the interactions between CD44 and HA are important in the successful homing of MSCs to sites of kidney injury [37].

To further elucidate the factors that might be responsible for the migration capacity of MSCs, Ponte *et al.* investigated the response of MSCs to 16 chemokines and growth factors *in vitro* and found that growth factors such as PDGF-AB and IGF-1, and to a lesser extent HGF and EGF, were the most potent chemoattractants of MSCs, rather than chemokines, including SDF-1 [38]. However, stimulation of MSCs with TNF-α significantly increased their migration capacity toward the chemokines RANTES (regulation on activation, normal T cell expressed and secreted), macrophage-derived chemokine, and SDF-1, which correlated with an increase of their membrane receptor expression on MSCs – chemokine receptor 3, chemokine receptor 4, and CXCR4 respectively [38]. These results demonstrate that the migration capacity of MSCs is controlled by a number of interactions between chemokines, growth factors, and receptors; and since MSC migration is increased when MSCs are primed with TNF-α, the homing of MSCs is likely dependent on the presence of inflammatory cues. In order for MSCs to enter the kidney, they must first adhere to and then migrate across the endothelium. The presence of inflammation has been reported to upregulate vascular cell adhesion molecule-1 on the endothelium [39], which binds MSCs via their expression of very late activation antigen (VLA)-4 [40], facilitating entry into the kidney interstitium.

The invasion of MSCs into damaged tissues also requires degradation of the basement membrane to which both endothelial and epithelial cells attach. This is likely mediated by protein-degrading enzymes; namely, MMPs. MMPs are a group of proteases that are responsible for the degradation of collagen and are regulated by TIMPs [41]. Transcript analysis of human BM-MSCs revealed that MMP-2, membrane-type 1 (MT1)-MMP, TIMP-1, and TIMP-2 are all expressed by MSCs, and when MMP-2, MT1-MMP, and TIMP-2 are knocked down the invasive capacity of MSCs is significantly hampered [42]. Furthermore, the presence of the inflammatory cytokines TGF-β1, IL-1β, and TNF-α served as chemoattractants for MSCs, significantly increasing their ability to migrate across basement membranes through the upregulation of MMP-2, MT1-MMP, and possibly MMP-9 [42].

While studies have demonstrated the successful homing of MSCs to the kidney, a "lung barrier" has been identified whereby MSCs initially accumulate in the small capillary beds of the lungs rather than the target organs when administered intravenously [43–45]. To overcome this, intra-arterial administration of MSCs [46,47] or the use of a vasodilator at the time of MSC administration have been used as a means of increasing MSC localization within the target organ [43,48].

However, identifying the location of MSCs after intravenous (IV) infusion will be important since the most commonly used route of MSC delivery in the clinic is likely to be by IV infusion [49]. Various methods to trace MSCs have been used, including magnetic resonance imaging of iron dextran-labeled MSCs [50–52], fluorescently tagged MSCs [53], and bioluminescent imaging [46]. Bioluminescence imaging is based on the principle that the firefly luciferase enzyme generates light, emitting photons when undergoing oxidation in the presence of adenosine-5′-triphosphate (ATP) [54]. The substrate for luciferase is D-luciferin, which can be injected intraperitoneally in experimental animals to activate the luciferase in an ATP-dependent manner [54]. In order to be detected, the photons released by the luciferase must pass through tissues and fluids before escaping through the skin of the animal, which is a

limitation of using any *in vivo* optical imaging to trace MSCs. Hemoglobin is responsible for absorbing blue and green light of wavelengths of 600 nm or below, and while some scattering of light occurs when it passes through tissue, the majority of red light (600–1100 nm) is emitted from the surface of the animal and is captured by a highly sensitive camera [54]. Studies have successfully utilized MSCs transduced with the luciferase gene [55] in order to determine their *in vivo* distribution, which offers advantages over other imaging techniques. For example, magnetic resonance imaging of MSCs can result in the identification of nonviable cells or even cells other than the labeled MSCs because of iron shedding [46]. However, because the generation of photons from the luciferase gene is dependent on ATP, only metabolically active cells will emit light and be detected using bioluminescence [54].

An additional advantage of this technique is that mammals do not emit a background bioluminescent signal; therefore, the sensitivity of bioluminescent tracking remains high. Importantly, the luciferase gene can be activated multiple times over an extended period of time; therefore, animals can be imaged over many time points and the localization of the MSCs identified, thus reducing the number of animals required. Conversely, fluorescence-based imaging techniques have inherent problems with background autofluorescence in mammalian tissue, significantly affecting the signal-to-noise ratio [54].

Our recent studies in a fibrotic model of experimental kidney injury have utilized bioluminescence imaging and demonstrated that venous administration of luciferase/green fluorescent protein (GFP)-tagged MSCs could overcome the "lung barrier" and home to the injured kidney (Figure 51.2).

Imaging at 1, 24 and 36 h after MSC administration to both injured and sham-operated mice demonstrated that MSCs were present only in the lungs of sham-operated mice, but in both the lungs and the kidney of injured mice after 1 h. By 24 h, however, MSCs were localized in the injured kidneys only (Figure 51.2a) and not in sham controls (Figure 51.2b) [56].

These results indicate that, following intravenous administration, MSCs are capable of homing out of the lungs and into the injured kidney. Of note also is that the MSCs remained for longer and in higher numbers in an inflammatory setting, as seen in

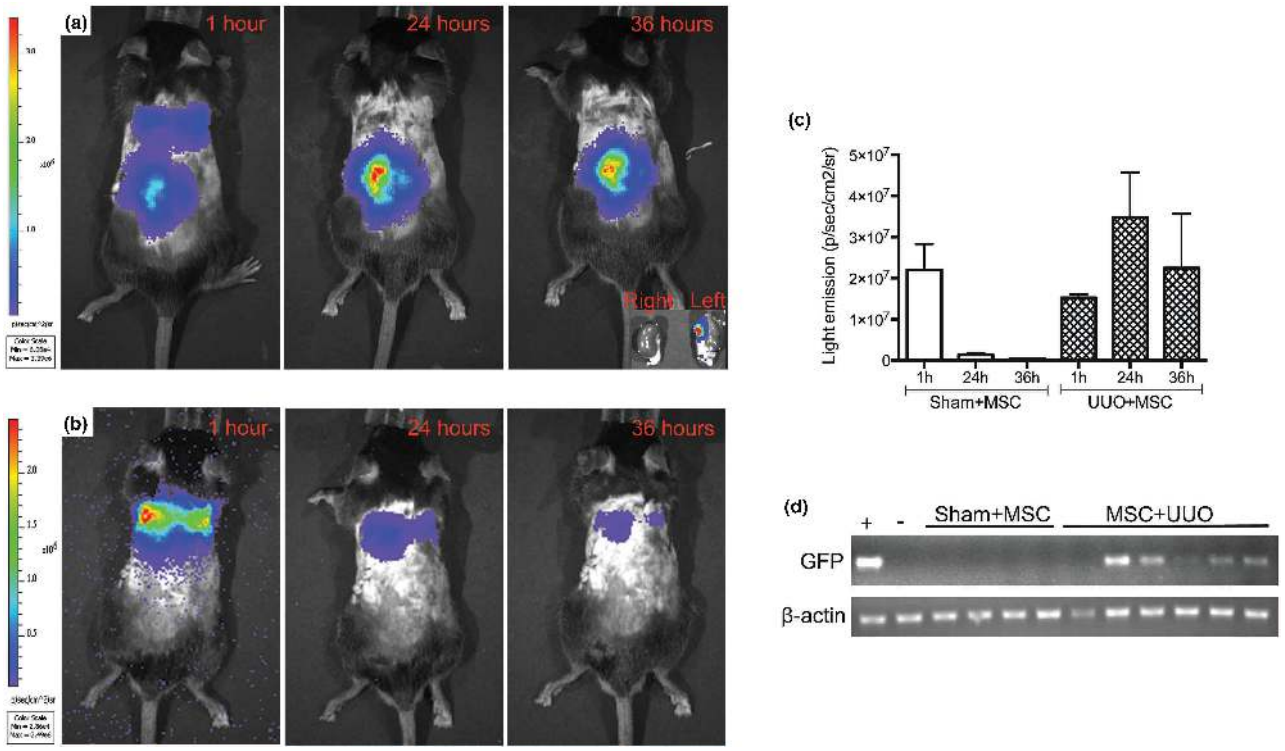



**Figure 51.2** MSCs can home to the injured kidney after intravenous transplantation. Luciferin/GFP+-tagged MSCs can be utilized to visualize MSC distribution *in vivo*. Luciferin requires ATP to produce photons and emit light; therefore, only viable MSCs will be detected using this method. Additionally, the amount of light produced is directly proportional to the amount of MSCs present. (a) After ureteric obstruction, MSCs can be observed in both the lung and the obstructed kidney as early as 1 h post MSC administration, yet after 24 h a strong bioluminescent signal originates from the area of the obstructed kidney only, where it remains for up to 36 h. Excision of the kidneys revealed that MSCs were located only in the injured kidney, and not the uninjured control. (b) In comparison, MSCs do not home to the kidneys in sham-operated controls. Instead, MSCs home to the lungs, where they remain for up to 36 h yet are hardly detectable. (c) When the bioluminescent signal is quantified from the area of the kidneys in injured mice, and the area of the lungs in sham-operated mice, the differences in light emission can be compared. (d) Polymerase chain reaction of genomic DNA extracted from whole kidneys showed that the GFP gene was present in MSC-treated groups, and not sham-operated controls. UUO, unilateral ureteric obstruction. (See color plate section.) *Source*: adapted from Huuskes 2015 [56].

Figure 51.2c: at 36 h the bioluminescent signal had almost completely diminished over the area of the lungs in sham-operated animals, but was still present in the injured kidney [56], suggesting that inflammation is an important cue for MSC homing and localization within the injured kidney. Similarly, in an experimental model of IR injury, MSCs were undetectable in sham-operated mice by 72 h, yet homed out of the lungs and engrafted into both unilateral and bilateral kidneys in IR mice [33].

This increased capacity of MSCs to migrate out of the lungs and into injured kidneys may be due to MSCs' ability to produce or influence MMP activity [42]. MSCs can stimulate MMP production from cultured cardiac fibroblasts [57], and increased MMP-2 activity is likely the major mechanism for reducing lung fibrosis when administering umbilical cord MSCs [58]. Of further significance, the migration of MSCs into injured tissues is reliant on MMP-2-dependent degradation of the basement membrane [42,59].

## 51.4 Mechanisms of kidney injury and capacity for repair

A growing body of evidence suggests that MSCs can attenuate or reverse kidney damage, dependent on the severity of injury. However, the efficacy of MSCs is limited following a more chronic insult, with the accumulation of ECM protein leading to the establishment of fibrosis. The challenge of reversing kidney fibrosis is, in part, due to the difficulties in adequate cell replacement in order to preserve structure and improve function. The highly specialized and complex structural architecture of the kidney makes cell replacement difficult. The functional unity of the kidney consists of nephrons (containing a glomerulus), which are mainly responsible for filtration, and a series of connecting tubules, which are responsible for reabsorption. The tubulointerstitium surrounding the glomerulus comprises approximately 80% of kidney volume, predominately containing the renal tubules, and performs the majority of the critical functions of the kidney [60]. As such, it has a high energy demand and is thus susceptible to injury resulting from ischemic or fibrotic environments [61].

After transient injury the kidney has a remarkable ability to undergo endogenous repair, yet the underlying mechanisms are still under debate. Cochrane *et al.* [62] demonstrated the regenerative potential of the adult mouse kidney by inducing kidney injury using a unilateral ureteric obstruction (UUO) model and then reversing the ureteric ligation (RUUO) after 10 days. Six weeks after the reversal of injury, kidney fibrosis and macrophage infiltration were significantly decreased, and this correlated with an increase in GFR [62]. The origins of the cells that are responsible for this endogenous repair are an ongoing debate; however, data have demonstrated that, rather than a kidney stromal/progenitor cell inducing tubular regeneration, existing tubule cells undergo self-duplication whereby they dedifferentiate and proliferate to replace damaged tubular epithelial cells [63]. Rinkevich *et al.* [64] confirmed previous findings by Humphreys *et al.* [63], but further elucidated that tubular regeneration happens in a fate-restricted fashion. By inducing IR injury in rainbow mice that express green, yellow, blue, or red epithelial reporters, long segments of epithelial cells expressing a single colored reporter were found in the cortex, medulla, and papilla 2 months after injury. This suggests that each segment of the tubule is responsible for its own repair through proliferation of existing tubular cells; for example, cells isolated from the papilla only formed collecting ducts and not proximal or distal tubules [64]. Interestingly, by 7 months, single clones were also found in long segments of tubules without changes in renal mass, indicating that tubulogenesis continues into adulthood with a subset of tubular epithelial cells responsible for this maintenance [64].

Despite endogenous repair in the short term, there is an inextricable link between the severity of disease and the ability for the kidney to undergo endogenous repair. Studies have demonstrated that UUO in neonatal mice not only impairs the growth and development of the kidney, but that fibrosis persists even 28 days after RUUO [65]. Further studies by the same group showed that obstructing one kidney in neonatal mice had a significant effect on the contralateral unobstructed kidney, resulting in delayed maturation and atubular glomeruli [66]. More significantly, a growing number of patients are progressing to CKD after acute kidney injury. Hence, there is urgent need for therapeutic agents that can promote endogenous repair of the kidney and restore renal function.

## 51.5 Kidney injury and repair in balance with fibrosis

A hallmark of all kidney diseases is fibrosis, which results from aberrant wound healing. Inflammation plays an important role in initiating the fibrotic pathway, as the release of TGF-β from local and infiltrating immune cells activates fibroblasts, transforming them into myofibroblasts, which are responsible for the extensive collagen production in the renal parenchyma that ultimately leads to fibrosis [67] (Figure 51.1). Myofibroblasts are identified by their expression of α-SMA, and studies have demonstrated that DNA methylation might be responsible for sustaining fibroblast activation [68]. Fibroblasts isolated from mouse kidneys exposed to TGF-β1 demonstrated hypermethylation of the promoter region of the transcription factor RAS protein activator like 1 (Rasal 1) after 5 days of culture. The hypermethlyation of Rasal 1 was concurrent with extensive fibrogenesis and was shown to be dependent on DNA-methyltransferase (Dnmt) 1 as Dnmt knockdown mice were resistant to kidney fibrosis [69]. The exact mechanism of how Dnmt can hypermethylate genes is yet to be elucidated. The origin of myofibroblasts during kidney fibrosis has been the subject of considerable debate [70,71]. Many investigations have suggested that myofibroblasts are derived from cells undergoing

epithelial-to-mesenchymal transition, or endothelial-to-mesenchymal transition, or that pericytes and BM recruitment give rise to these cells [72–74]. However, data using transgenic knockout mice demonstrated that pericytes do not contribute to myofibroblast accumulation in the injured kidney and that, together, epithelial-to-mesenchymal transition and endothelial-to-mesenchymal transition only contribute approximately 15% of the myofibroblast pool [75]. Of further significance is that the proliferation of local fibroblasts and subsequent conversion into myofibroblasts contributed around half of the total myofibroblast accumulation within the kidney, while the migration and differentiation of BM cells was found to be responsible for an additional 35% of the myofibroblast pool [75]. Conversely, a study has demonstrated that circulating infiltrating BM cells do not contribute to the myofibroblast pool [75]; rather, tissue-resident Gli1$^+$ (glioma-associated antigen 1) progenitor cells transformed into myofibroblasts, while ablation of the Gli1$^+$ pool resulted in the amelioration of the fibrosis in lung, liver, heart, and kidney damage [76]. Understanding the mechanisms that perpetuate fibrosis is therefore important for the development of novel therapeutic agents that can target progression of disease.

The deposition of ECM widens the interstitial space, increasing the distance between the tubules and capillaries, thereby limiting the diffusion of oxygen through the interstitium and is a major contributor to the progression of kidney disease [77]. In a model of UUO, renal expression of EGF was significantly decreased, further exacerbating ischemia and leading to tubular apoptosis and capillary loss [78]. The vascularization of the kidney after injury can be determined using immunohistochemical (IHC) techniques with a marker for endothelial cells such as von Willebrand factor or CD31 [79,80], or with genetically modified animals with reporter genes for endothelial cells [81]. These methods, however, do not provide information about the perfusion state of capillaries and may give an overrepresentation of surviving capillaries as embolized and collapsed capillaries remain identified. The identification of endothelial cells has also been determined using a novel technique called fluorescent migroangiography (FMA) and has been validated with a tdTomato genetic reporter of endothelial cells [82]. (tdTomato protein is a very bright red fluorescent protein). FMA is a technique in which experimental animals are perfusion fixed with an agarose/fluorescent microbead mixture, after which the organs can be harvested and imaged, allowing the microvascular architecture to be quantified and reconstructed into a three-dimensional image [83]. The comparison of FMA and IHC markers to identify endothelial cells was made in a moderate and severe model of IR injury, and results confirmed that while some capillaries are positive for IHC markers, they may not be well perfused [82]. Thus, FMA may provide a more sensitive method for determining the difference in moderate and severe vascular changes in response to IR injury [82]. Hence, a critical component of the regenerating kidney is to also target the interstitial vasculature.

MSCs have demonstrated vasculogeneic and angiogenic properties in experimental models of acute kidney injury. This was first recognized by Tögel *et al.* [8] when they suspended human placental alkaline phosphatase$^+$ (hPAP) MSCs in Matrigel and administered them subcutaneously to rats. After 7 days the Matrigel was removed and hPAP cells were stained to reveal vascular-like structures *in vivo*. Furthermore, gene analysis revealed that MSCs express similar vascular tropic factors to those by endothelial cells, including VEGF-C, bFGF, heparin-binding EGF-like growth factor, and TNF-α, as well as high expression levels of IGF-1, VEGF-A, and VEGF-D [8].

## 51.6 Mesenchymal stromal cells as delivery tools for therapies

Since studies have demonstrated that MSCs can home to sites of inflammation and injury, it is plausible that MSCs could be used as a delivery tool for other therapeutic agents. As such, efforts have been made to increase MSC homing and efficacy through the transduction of MSCs with IGF-1 [84] and HGF-1 [85], with results demonstrating increased MSC engraftment, correlating with improved renal architecture compared with MSC therapy alone. Studies have previously shown that transducing MSCs with vesicles or growth factors can facilitate their homing into target organs [84,86,87], which is important in promoting MSC-mediated repair and also when considering the use of MSCs as therapeutic delivery tools [88].

Extracellular vesicles such as exosomes and microvesicles (MVs) have been considered as critical components for cell-to-cell communication. They differ based on a range of properties, such as size, surface markers, and biosynthesis, but are similar in the sense that that they are both able to deliver mRNA, microRNA (miRNA), and proteins to a target cell [89]. Extracellular vesicles are present in almost all cells, including MSCs [90]. Bruno *et al.* isolated and characterized MVs derived from MSCs and determined that CD44 and CD29 were essential for the fusion of MVs with tubular epithelial cells *in vitro*, but it was the transfer of RNA that was responsible for tubular cell proliferation and inhibition of apoptosis [90]. Further studies have described a protective effect of MSC-derived MVs in both acute and progressive models of kidney disease. Markers of kidney injury, such as tubular lesions, cell apoptosis, and blood urea nitrogen levels, decreased at early time points, while markers of CKD progression, such as serum proteinuria and tubulointerstitial fibrosis, were decreased 6 months after MV delivery [91]. MVs derived from endothelial progenitor cells (EPCs) have similar effects on kidney repair to those derived from MSCs, both of which can be reversed if RNAse is co-administered with the MVs [91,92]. Furthermore, genomic screening from MVs derived from EPCs showed 26 miRNAs specific to the MV that was not detected in the EPCs. These miRNAs differed from those isolated from fibroblasts: specifically, fibroblasts lacked miR-126 and miR-296, which are known

proangiogenic and antiapoptotic miRNAs [92], indicating that the source of MVs is critical in determining their ability to repair.

Studies with MSCs in experimental models of kidney injury are continuing to expand our knowledge of the mechanisms by which MSCs induce repair in acute and chronic models of kidney disease and the ways in which MSC efficacy can be enhanced (Table 51.1).

## 51.7 Clinical trials with mesenchymal stromal cells

Clinical trials with mesenchymal stromal cells are well underway for a variety of diseases, with safety consistently demonstrated [103] (Table 51.2). Tubulointerstitial fibrosis is not only a common phenotype observed in end-stage renal disease

**Table 51.1** Preclinical studies investigating MSCs derived from various sources to treat acute and chronic forms of kidney disease.

| Injury model | MSC source | Administration | Features | Reference |
|---|---|---|---|---|
| UUO | $1 \times 10^6$ BM-MSCs transfected with HGF. male Sprague Dawley rats | IV via tail vein into female Sprague Dawley rats | MSC localization into tubular epithelial cells of injured kidney; both MSCs and MSC-HGF showed decreased α-SMA expression | [85] |
| 45 min unilateral IR with contralateral nephrectomy | 30 μg of MV protein derived from human BM-MSCs | IV into male Sprague Dawley rats | Acute setting (2 days postinjury): decreased serum creatinine and BUN, increased tubule proliferation, decreased apoptosis. Chronic setting (6 months postinjury): decreased serum creatinine, BUN, proteinuria, and fibrosis | [91] |
| UUO | $1 \times 10^6$ human MSCs | Into the renal artery of male Sprague Dawley rats | MSC localization into renal cortex of injured kidney, increased epithelial cell preservation, decreased α-SMA, fibroblasts, and fibrosis | [47] |
| 5/6 nephrectomy | $0.5 \times 10^6$ BM-MSCs from male Sprague Dawley rats | IV via tail vein into male Sprague Dawley rats | MSC localization into injured kidney; improved renal function, increased morphological recovery, increased expression of Pax-2, bFGF, BMP-7, VEGF, and Tie-2; decreased tubular injury | [93] |
| UUO | $1 \times 10^6$ BM-MSCs from male Wistar rats | IV into the inferior vena cava | Decreased fibrosis, macrophage infiltration, and IFN-γ; increased expression of Foxp3 and IL-10 | [11] |
| Chronic aristo-lochic acid nephropathy | $2 \times 10^7$ BM-MSCs from male Wistar rats | IV via tail vein into female Wistar rats | MSC localization in the injured kidney; decreased TGF-β, BUN, creatinine, and proteinuria; increased morphological recovery and upregulation of HGF | [94] |
| Kidney transplantation: male Lewis rats received a kidney from male Fischer-344 rats | $0.5 \times 10^6$ $GFP^+$ BM-MSCs from male Sprague Dawley rats | IV via tail vein after 11 weeks of transplantation | No immunosuppression given posttransplant. No difference between treatment groups 1 week post MSCs; decreased tubular atrophy, fibrosis, lymphocytes, and macrophages | [95] |
| Anti-glomerular basement membrane glomerulo-nephritis | $3 \times 10^6$ human BM-MSCs | IV 4 days after postinjury in female WKY rats | MSC localization in the glomeruli and tubulointerstitium of injured kidney 5–48 h after administration but were not detectable by 7 days; decreased proteinuria, serum creatinine at late time points, decreased kidney weight, glomerular injury, apoptosis, TNF-α, IL-1β, IL-17, TGF-β, Col1 and Col3; increased IL-4, Foxp3, IL-10 | [96] |
| Cisplatin-mediated kidney injury | $2 \times 10^5$ BM-MSCs from male C57BL/6 J mice | IV into female C57B/6 J mice | MSC localization into peritubular areas of injured kidney preconditioning with IGF-1 increased MSC migration, improved renal function, and preserved morphology | [84] |
| 5/6 nephrectomy | $0.5 \times 10^6$ human adipose tissue-derived MSCs | IV via tail vein of male Sprague Dawley rats | MSC localization in the injured kidney; decreased BUN; increased morphological recovery, VEGF, Pax-2, and BMP-7 | [97] |
| Cisplatin-mediated kidney injury | $5 \times 10^5$ IV, $4 \times 10^6$ IP, or $1 \times 10^6$ renal subcapsular space BM-MSCs from C56BL/6 J $GFP^+$ mice | IV, IP, or renal subcapsular space | Improved renal function and morphological recovery regardless of route of administration | [98] |

(*continued*)

**Table 51.1** (*Continued*)

| Injury model | MSC source | Administration | Features | Reference |
|---|---|---|---|---|
| Cisplatin-mediated kidney injury | $1 \times 10^6$ BM-MSCs – male Sprague Dawley rats | IV into male Sprague Dawley rats | MSC localization in the peritubular space of injured kidneys; decreased BUN, serum creatinine; increased tubule proliferation and improved morphological recovery; decreased apoptosis and MAPKs phosphorylation | [99] |
| 5/6 nephrectomy | $1 \times 10^7$ MSCs transfected with GFP into Sprague Dawley rats | IV via tail vein at 4, 8, 12, and 16 weeks after injury in male Sprague Dawley rats | MSC localization in the glomeruli, tubular epithelial cells, and interstitial space at all time points; decreased fibrosis and glomerular injury; improved renal function at 4 and 8 weeks only | [100] |
| Type 1 diabetes mellitus | $1 \times 10^6$ BM-MSCs from male albino rats | IV into female albino rats | Improved renal function; increased Blc2 and Bax gene expression; decreased TGF-β and TNF-α expression | [101] |
| 40 min unilateral and 25 min bilateral IR | $1 \times 10^6$ human BM-MSCs | IV into C57BL/6 J mice | MSC localization in injured kidney; improved renal function; morphological recovery and tubular proliferation, decreased fibrosis and Kim-1 expression; increased MMP-9 expression | [33] |
| Polycystic kidney disease | $2.5 \times 10^5$ BM-MSCs from male Sprague Dawley rats | IA into the renal artery in female Sprague Dawley rats | No change in cyst number or size; blood pressure normalized; increased vascular density; decreased fibrosis | [102] |
| UUO | $1 \times 10^6$ human BM-MSCs | IV into C57BL/6 J mice | MSC localization into the obstructed kidney; increased morphological preservation; decreased fibrosis; increased MMP-2 | [56] |

Bax: blc-2-like protein 4; Bcl: B cell lymphoma; BMP: bone morphogenetic protein; BUN: blood urea nitrogen; Col: collagen; Foxp3: forkhead box p3; IP: intraperitoneal; IA: intra-arterial; Kim: kidney injury molecule; MAPK: mitogen-activated protein kinase; Pax-2: paired box gene 2; Tie-2: angiopoietin receptor-2.

**Table 51.2** Published clinical trials investigating the use of MSCs derived from various sources in the setting of solid organ kidney transplantation.

| Timing | Cell number and source | Patient number | Main results | Reference |
|---|---|---|---|---|
| 7 days after kidney transplant | Autologous BM-MSCs $1.7 \times 10^6$/kg and $2 \times 10^6$/kg | 2 | Restricted memory T cell expansion; enlarged regulatory T cell population; renal insufficiency from 7 to 14 days after MSC infusion; good graft function at follow-up 360 and 180 days posttransplant | [104] |
| TIP using MSCs | Various cell numbers of adipose-tissue-derived MSCs and hematopoietic stem cells from kidney donors | 916<br>Group 1: MSCs and HLA-matched<br>Group 3: MSCs and not HLA-matched<br>310 controls<br>Group 2: HLA-matched<br>Group 4: not HLA-matched | Patients receiving TIP (groups 1 and 3) had 1- and 4-year follow-up outcomes similar to those of HLA-matched controls (group 2). Groups 1–3 had increased patient survival and graft function compared with group 4. A percentage of patients in groups 1 and 3 (17–21%) had rejection episodes and were placed on conventional triple immune suppression, yet a further 5% developed chronic rejection and half of these lost the graft. Conclusion: MSCs are feasible for induction therapy | [105] |
| Induction therapy using MSCs. Kidney transplants from living related donors | Autologous BM-MSCs $1–2 \times 10^6$/kg | 159<br>Group A: MSCs + standard CNI<br>Group B: MSCs + low-dose CNI<br>Group C: Controls | Increased GFR in groups A and B versus group C, but only for the first month posttransplant; early time points showed that groups A and B had decreased acute rejection, but at 1-year follow-up no difference between any groups was observed; opportunistic infection decreased in groups A and B at 1-year follow-up. Conclusion: MSCs were safe and well tolerated | [49] |

**Table 51.2** (*Continued*)

| Timing | Cell number and source | Patient number | Main results | Reference |
|---|---|---|---|---|
| 1 day pretransplant | Autologous BM-MSCs $2 \times 10^6$/kg | 8 (2 subjects and 6 controls) | Decreased donor-specific cytotoxic and $CD8^+$ T cells. MSC infusion did not affect the graft. MSCs had comparable immunomodulatory effects as standard induction therapy. Follow-up at 1 year | [106] |
| If signs of rejection on renal biopsy at 4 weeks and at 6 months posttransplant | Two doses of autologous BM-MSCs $1–2 \times 10^6$/kg 7 days apart | 6 | Decreased donor-specific PBMC proliferation; resolution of tubulitis; MSCs were well tolerated | [107] |
| Patients who had already received pretransplant MSCs and who had stable graft function at >2 years with serum creatinine <2 | Allogeneic hematopoietic stem cells $17.5 \pm 5.5 \times 10^8$/kg. In 55 patients, donor adipose-tissue-derived MSCs were additionally administered | 76 | All immune-suppressive medication except prednisone was withdrawn from patients, with no adverse effects. No adverse rejection episodes. Follow-up $5.5 \pm 3$ years | [108] |
| Pretransplant infusion after living related donor kidney transplant and 1 month posttransplant | Kidney-donor-derived BM-MSCs delivered twice: $5 \times 10^5$/kg pretransplant and 1 month later $2 \times 10^6$/kg | 12 (6 subjects and 6 controls) | Standard immune suppression was able to be reduced in MSC group; no chimerism detected at 3 months; MSC recipients showed significantly higher B cell levels at 3 months compared with controls; MSCs were safe and well tolerated. Follow-up 1 year | [109] |
| Induction therapy 16 days pretransplant | Donor adipose-tissue-derived MSCs ($2.5 \times 10^2$/μL – total of 3 mL infused) and BM-HSCs ($2.18 \times 10^4$/μL – total of 99 mL infused) | 1 | Stable graft function with no immunosuppressive therapy from 6 months onwards. Follow-up to 35 months. | [110] |

CNI: calcineurin inhibitor; HLA: human leukocyte antigen; TIP: tolerance induction protocol.

(ESRD), but is also a common cause for renal allograft rejection [111]; as such, a number of ongoing clinical trials are investigating the use of MSCs in the kidney transplant setting [107] (see Chapter 56). While long-term studies are yet to be concluded, early reports demonstrate that MSC therapy is well tolerated as a form of induction therapy [49] and can also reduce fibrosis when subclinical rejection has started [112]. It is important to note, however, that the timing of MSC administration is a key factor in determining the outcome of such an approach, since MSC administration pretransplant restricted T cell proliferation, but when they were administered 7 days posttransplant they induced graft dysfunction [104]. Of importance, BM-MSCs from patients with ESRD had similar morphology, growth characteristics, mRNA expression profiles, and immunosuppressive properties to BM-MSCs isolated from healthy individuals [112]. This is important when considering autologous MSCs as a potential treatment.

With the discovery of MSCs, and more recently of embryonic stem cells and induced pluripotent stem cells, regenerative medicine has the potential to repair damaged tissues *in vivo*. Ultimately, patient-specific differentiated stromal cell-derived progeny may be used to home to and/or engraft at the site of injury and differentiate into specialized functional cells, replacing scar tissue [113]. However, this is proving to be challenging due, in part, to the complex interplay of regulatory mechanisms governing cell replacement and tissue remodeling. In addition, the reversibility of fibrotic tissue damage is dependent on the progression of the injury. Cell-based therapy is likely to be advantageous when used as a complement to existing therapies [114]. Despite the regenerative capacity in settings of acute kidney disease, the development of fibrosis in chronic disease models has been shown to hamper MSC survival and limits integration of transplanted cells into the host tissue, thus significantly impacting MSC-dependent repair and restoration of function [115–118]. Thus, the use of MSCs in conjunction with an antifibrotic agent, such as relaxin, may enhance cell viability and increase MSC-mediated tissue repair.

## 51.8 The antifibrotic functions of relaxin

Relaxin is an endogenous hormone first identified in 1926, when it was shown to be important in the relaxation of pelvic ligaments during pregnancy [119]. More recently, humans have been shown to express three relaxin peptides (H1, H2 and H3),

with a further four insulin-like peptides classified as members of the relaxin peptide family of hormones and distinguished from insulin and IGF by their ability to act through G-protein-coupled receptors [120].

Studies with relaxin-null and relaxin family peptide receptor (RXFP1)-null mice have demonstrated that endogenous relaxin plays an important homeostatic role in collagen regulation, as age-related fibrosis is often observed in these knockout mice as well as an inability to undergo sexual maturation [121–126]. Furthermore, in experimental models of kidney disease, relaxin-null mice had significantly higher fibrosis scores than wild-type mice; however, this was normalized with the administration of exogenous relaxin (serelaxin) [127]. The administration of exogenous serelaxin, a recombinant form of human relaxin 2, in a variety experimental models of renal disease, including papillary necrosis, renal mass reduction, and, more recently, cisplatin toxicity and IR injury, has abrogated disease-induced fibrosis [73,128–132]. In a UUO model of fibrosis, exogenously administered serelaxin suppressed TGF-β1 expression, mothers against decapentaplegic homolog 2 (Smad2) phosphorylation, and myofibroblast differentiation, which resulted in a marked reduction in renal collagen accumulation [73]. Importantly, serelaxin only targets TGF-β-induced fibrosis; thus, basal levels of collagen are unaffected [133].

In a number of cell culture and animal models of disease (reviewed in [133]) serelaxin was able to antagonize the actions of TGF-β by inhibiting Smad2 phosphorylation, a protein involved in the regulation of TGF-β1 signal transduction and activity [134]. Through this inhibition serelaxin optimally inhibits myofibroblast differentiation and subsequent collagen synthesis, while promoting MMP activity when circulating levels mimicked physiological serum concentrations during pregnancy [134]. An extension of these studies confirmed that serelaxin acted via RXFP1 and the nitric oxide (NO) pathway, specifically via a neuronal NO synthase (nNOS)–NO–cyclic guanosine monophosphate (cGMP)-dependent mechanism to disrupt Smad2 phosphorylation as a means of interfering with TGF-β1 signal transduction [135]. Furthermore, a secondary mechanism of the antifibrotic action of serelaxin was found that promoted collagen degradation by suppressing TGF-β1 activity and increased the availability of iNOS, and therefore NO, which in turn upregulated MMP activity [136].

In addition to its potent antifibrotic actions, relaxin contributes to the homeostasis of vascular function in many organs. Administration of relaxin generally has a vasodilatory effect on the vasculature of most organ systems. In the kidney this is complemented by a decrease in vascular resistance and an increased GFR [137]. Serelaxin has shown angiogenic properties that can contribute to blood vessel formation in diabetic skin wounds [138] and ischemic wound sites in the heart [139], while antiapoptotic actions have been observed in the injured heart [140] and kidney [73], suggesting that relaxin may be beneficial in the attenuation of scarring.

## 51.9 Combination therapy using mesenchymal stromal cells and relaxin

We have demonstrated that delivery of exogenous serelaxin improves MSC-induced renal repair and promotes protection from fibrotic injury [56] (Figure 51.1). In a UUO model of kidney fibrosis this combined therapeutic approach resulted in a significant decrease in collagen content compared with either MSCs or serelaxin delivered alone [56]. Of further significance, the synergistic actions of MSCs and serelaxin in protecting the obstructed kidney from fibrosis were likely due to their combined effect on the matrix degradation pathway, as only the combined delivery of both MSCs and serelaxin resulted in a significant upregulation of MMP-2 [56]. Macrophage infiltration and the collagen synthesis pathway were also investigated in this study. However, there was no apparent effect of combination therapy on infiltrating macrophages, α-SMA, or TGF-β expression [56]. Importantly, *in vitro* studies suggest for the first time that serelaxin may be influencing MSCs, as both the migration and proliferation capacity of MSCs were significantly increased when serelaxin was present [56]. Therefore, manipulating the inflammatory kidney microenvironment through the synergistic delivery of antifibrotic agents may aid in MSC efficacy and viability, and thus improve renal function over the long term.

Supportive evidence that stromal cell viability can be increased in the presence of serelaxin originated from heart failure models in rats and pigs. In a myocardial infarction model of heart failure, administration of serelaxin was found to prevent the progression of fibrosis by 30–40% in the infarct region and promoted angiogenesis while inhibiting cadiomyocyte apoptosis [140]. Seemingly, these actions of relaxin were what facilitated the beneficial effects of skeletal myoblasts engineered to express relaxin when administered in a rat chronic heart failure model [141]. After this combination treatment, postinfarct fibrosis progression was attenuated, while an improvement of cardiac function was observed. Previous studies by the same group suggested that stromal cell viability was increased when relaxin was present [139].

## 51.10 Conclusions

The development of fibrosis is the inevitable consequence of ESRD, regardless of etiology. Current treatment options for patients living with CKD can only slow the progression of the disease, with a substantial number still progressing to ESRD, for which dialysis or solid organ transplantation remain the only treatment options. Recent advances in our understanding of MSC recruitment and capability in response to microenvironmental cues raise new and exciting therapeutic possibilities to attenuate or conceivably reverse progressive renal disease in the context of fibrosis. Future therapeutic approaches can be

envisaged that capitalize on a cell-based therapy in combination with antifibrotics or proteins such as relaxin, to dampen inflammation, provide immune modulation, and promote normal tissue remodeling. With MSCs and relaxin already individually in clinical trials in a number of other indications, future options may include the combination of both MSCs and relaxin as an alternative for patients that respond poorly to currently available treatment. Alternatively, they could be used in the solid organ transplantation setting, to extend the life of the graft by preventing the chronic rejection that results from fibrosis. In addition, the advent of identifying reliable biomarkers that provide a good indication of the progression of kidney disease will aid in the optimal timing of MSC delivery together with existing or novel therapeutic agents.

## References


1 A. F. Wise and S. D. Ricardo. Mesenchymal stem cells in kidney inflammation and repair. *Nephrology (Carlton)*, 2012;**17**(1):1–10.
2 K. O'Donoghue and J. Chan. Human fetal mesenchymal stem cells. *Curr Stem Cell Res Ther* 2006;**1**(3):371–386.
3 L. da Silva Meirelles. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**(11):2204–2213.
4 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.
5 F. E. Tögel and C. Westenfelder. Mesenchymal stem cells: a new therapeutic tool for AKI. *Nat Rev Nephrol* 2010;**6**(3):179–183.
6 M. E. J. Reinders, W. E. Fibbe, and T. J. Rabelink. Multipotent mesenchymal stromal cell therapy in renal disease and kidney transplantation. *Nephrol Dial Transplant* 2010;**25**(1):17–24.
7 M. P. De Miguel, S. Fuentes-Julián, A. Blázquez-Martínez, *et al.* Immunosuppressive properties of mesenchymal stem cells: advances and applications. *Curr Mol Med* 2012;**12**(5):574–591.
8 F. Tögel, Z. Hu, K. Weiss, *et al.* Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 2005;**289**(1):F31–F42.
9 B. Bi, R. Schmitt, M. Israilova, *et al.* Stromal cells protect against acute tubular injury via an endocrine effect. *J Am Soc Nephrol* 2007;**18**(9):2486–2496.
10 P. P. Semedo, C. G. C. Palasio, C. D. C. Oliveira, *et al.* Early modulation of inflammation by mesenchymal stem cell after acute kidney injury. *Int Immunopharmacol* 2009;**9**(6):677–682.
11 Y.-L. Liu, Y.-D. Wang, F. Zhuang, *et al.* Immunosuppression effects of bone marrow mesenchymal stem cells on renal interstitial injury in rats with unilateral ureteral obstruction. *Cell Immunol* 2012;**276**(1):144–152.
12 S. Beyth, Z. Borovsky, D. Mevorach, *et al.* Human mesenchymal stem cells alter antigen-presenting cell maturation and induce T-cell unresponsiveness. *Blood* 2005;**105**(5):2214–2219.
13 X.-X. Jiang, Y. Zhang, B. Liu, *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 2005;**105**(10):4120–4126.
14 F. Djouad, L.-M. Charbonnier, C. Bouffi, *et al.* Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. *Stem Cells* 2007;**25**(8):2025–2032.
15 G. M. Spaggiari, H. Abdelrazik, F. Becchetti, and L. Moretta. MSCs inhibit monocyte-derived DC maturation and function by selectively interfering with the generation of immature DCs: central role of MSC-derived prostaglandin $E_2$. *Blood* 2009;**113**(26):6576–6583.
16 P. A. Sotiropoulou, S. A. Perez, A. D. Gritzapis, *et al.* Interactions between human mesenchymal stem cells and natural killer cells, *Stem Cells* **200624**(1):74–85.
17 G. M. Spaggiari, A. Capobianco, S. Becchetti, *et al.* Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. *Blood* 2006;**107**(4):1484–1490.
18 M. J. Crop, C. C. Baan, S. S. Korevaar, *et al.* Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation* 2009;**87**(6):896–906.
19 S. Asari, S. Itakura, K. Ferreri, *et al.* Mesenchymal stem cells suppress B-cell terminal differentiation. *Exp Hematol* 2009;**37**(5):604–615.
20 A. Corcione, F. Benvenuto, E. Ferretti, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**(1):367–372.
21 R. Meisel, A. Zibert, M. Laryea, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**(12):4619–4621.
22 M. Krampera, L. Cosmi, R. Angeli, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**(2):386–398.
23 E. Traggiai, S. Volpi, F. Schena, *et al.* Bone marrow-derived mesenchymal stem cells induce both polyclonal expansion and differentiation of B cells isolated from healthy donors and systemic lupus erythematosus patients. *Stem Cells* 2008;**26**(2):562–569.
24 M. M. Rosado, M. E. Bernardo, M. Scarsella, *et al.* Inhibition of B-cell proliferation and antibody production by mesenchymal stromal cells is mediated by T cells. *Stem Cells Dev* 2015;**24**:93–103.
25 G. Xu, Y. Zhang, L. Zhang, *et al.* C/EBPβ mediates synergistic upregulation of gene expression by interferon-γ and tumor necrosis factor-α in bone marrow-derived mesenchymal stem cells. *Stem Cells* 2009;**27**(4):942–948.
26 S. Aggarwal and M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.
27 G. J. Ko, C.-S. Boo, S. K. Jo, *et al.* Macrophages contribute to the development of renal fibrosis following ischaemia/reperfusion-induced acute kidney injury. *Nephrol Dial Transplant* 2008;**23**(3):842–852.
28 R. D. Stout, C. Jiang, B. Matta, *et al.* Macrophages sequentially change their functional phenotype in response to changes in microenvironmental influences. *J Immunol* 2005;**175**(1):342–349.
29 S. D. S. Ricardo, H. H. van Goor, and A. A. A. Eddy. Macrophage diversity in renal injury and repair. *J Clin Invest* 2008;**118**(11):3522–3530.
30 H.-J. Anders and M. Ryu. Renal microenvironments and macrophage phenotypes determine progression or resolution of renal inflammation and fibrosis. *Kidney Int* 2011;**80**(9):915–925.
31 K. Németh, A. Leelahavanichkul, P. S. T. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent

reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med* 2009;**15**(1):42–49.
32 M. A. Alikhan, C. V. Jones, T. M. Williams, *et al.* Colony-stimulating factor-1 promotes kidney growth and repair via alteration of macrophage responses. *Am J Pathol* 2011;**179**(3):1243–1256.
33 A. F. Wise, T. M. Williams, M. B. G. Kiewiet, *et al.* Human mesenchymal stem cells alter macrophage phenotype and promote regeneration via homing to the kidney following ischemia-reperfusion injury. *Am J Physiol Renal Physiol* 2014;**306**(10):F1222–F1235.
34 G. Brooke, M. Cook, C. Blair, *et al.* Therapeutic applications of mesenchymal stromal cells. *Sem Cell Dev Biol* 2007;**18**(6):846–858.
35 F. Tögel, J. Isaac, Z. Hu, *et al.* Renal SDF-1 signals mobilization and homing of CXCR4-positive cells to the kidney after ischemic injury. *Kidney Int* 2005;**67**(5):1772–1784.
36 P. A. Conget and J. J. Minguell. Phenotypical and functional properties of human bone marrow mesenchymal progenitor cells. *J Cell Physiol* 1999;**181**(1):67–73.
37 M. B. Herrera, B. Bussolati, S. Bruno, *et al.* Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. *Kidney Int* 2007;**72**(4):430–441.
38 A. L. Ponte, E. Marais, N. Gallay, *et al.* The *in vitro* migration capacity of human bone marrow mesenchymal stem cells: comparison of chemokine and growth factor chemotactic activities, *Stem Cells* 2007;**25**(7):1737–1745.
39 S. Klahr and J. Morrissey. Obstructive nephropathy and renal fibrosis. *Am J Physiol Renal Physiol* 2002;**283**(5):F861–F875.
40 A. Peled, O. Kollet, T. Ponomaryov, *et al.* The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human $CD34^+$ cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. *Blood* 2000;**95**(11):3289–3296.
41 R. Visse and H. Nagase. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. *Circ Res* 2003;**92**(8):827–839.
42 C. Ries, V. Egea, M. Karow, *et al.* MMP-2, MT1-MMP, and TIMP-2 are essential for the invasive capacity of human mesenchymal stem cells): differential regulation by inflammatory cytokines. *Blood* 2007;**109**(9):4055–4063.
43 S. Schrepfer, T. Deuse, H. Reichenspurner, *et al.* Stem cell transplantation: the lung barrier. *Transplant Proc* 2007;**39**(2):573–576.
44 H. Kanazawa, Y. Fujimoto, T. Teratani, *et al.* Bone marrow-derived mesenchymal stem cells ameliorate hepatic ischemia reperfusion injury in a rat model. *PLoS ONE* 2011;**6**(4):e19195.
45 V. Burst, F. Pütsch, T. Kubacki, *et al.* Survival and distribution of injected haematopoietic stem cells in acute kidney injury. *Nephrol Dial Transplant* 2013;**28**(5):1131–1139.
46 F. Tögel, Y. Yang, P. Zhang, *et al.* Bioluminescence imaging to monitor the *in vivo* distribution of administered mesenchymal stem cells in acute kidney injury. *Am J Physiol Renal Physiol* 2008;**295**(1):315–321.
47 H. Asanuma, B. A. Vanderbrink, M. T. Campbell, *et al.* Arterially delivered mesenchymal stem cells prevent obstruction-induced renal fibrosis. *J Surg Res* 2011;**168**(1):e51–e59.
48 G. Ali, S. Mohsin, M. Khan, *et al.* Nitric oxide augments mesenchymal stem cell ability to repair liver fibrosis. *J Transl Med* 2012;**10**:75.
49 J. Tan, W. Wu, X. Xu, *et al.* Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* 2012;**307**(11):1169–1177.
50 C. Lange, F. Tögel, H. Ittrich, *et al.* Administered mesenchymal stem cells enhance recovery from ischemia/reperfusion-induced acute renal failure in rats. *Kidney Int* 2005;**68**(4):1613–1617.
51 A. J. El Haj, J. R. Glossop, H. S. Sura, *et al.* An *in vitro* model of mesenchymal stem cell targeting using magnetic particle labelling. *J Tissue Eng Regen Med* 2015;**9**:724–733.
52 R. Moghadasali, M. Azarnia, M. Hajinasrollah, *et al.* Intra-renal arterial injection of autologous bone marrow mesenchymal stromal cells ameliorates cisplatin-induced acute kidney injury in a rhesus macaque mulatta monkey model. *Cytotherapy* 2014;**16**(6):734–749.
53 E. E. Graves, R. Weissleder, and V. Ntziachristos. Fluorescence molecular imaging of small animal tumor models. *Curr Mol Med* 2004;**4**(4):419–430.
54 C. H. Contag and M. H. Bachmann. Advances in *in vivo* bioluminescence imaging of gene expression. *Annu Rev Biomed Eng* 2002;**4**:235–260.
55 N. L. Payne, G. Sun, C. McDonald, *et al.* Distinct immunomodulatory and migratory mechanisms underpin the therapeutic potential of human mesenchymal stem cells in autoimmune demyelination. *Cell Transplant* 2013;**22**(8):1409–1425.
56 B. M. Huuskes, A. F. Wise, A. J. Cox, *et al.* Combination therapy of mesenchymal stem cells and serelaxin effectively attenuates renal fibrosis in obstructive nephropathy. *FASEB J* 2015;**29**(2):540–553.
57 C. Mias, O. Lairez, E. Trouche, *et al.* Mesenchymal stem cells promote matrix metalloproteinase secretion by cardiac fibroblasts and reduce cardiac ventricular fibrosis after myocardial infarction. *Stem Cells* 2009;**27**(11):2734–2743.
58 Y. Moodley, D. Atienza, U. Manuelpillai, *et al.* Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury. *Am J Pathol* 2009;**175**(1):303–313.
59 T. Tondreau, N. Meuleman, B. Stamatopoulos, *et al. In vitro* study of matrix metalloproteinase/tissue inhibitor of metalloproteinase production by mesenchymal stromal cells in response to inflammatory cytokines: the role of their migration in injured tissues. *Cytotherapy* 2009;**11**(5):559–569.
60 K. S. Hodgkins and H. W. Schnaper. Tubulointerstitial injury and the progression of chronic kidney disease. *Pediatr Nephrol* 2012;**27**(6):901–909.
61 J. V. J. Bonventre and J. M. J. Weinberg, Recent advances in the pathophysiology of ischemic acute renal failure. *J Am Soc Nephrol* 2003;**14**(8):2199–2210.
62 A. L. A. Cochrane, M. M. M. Kett, C. S. C. Samuel, *et al.* Renal structural and functional repair in a mouse model of reversal of ureteral obstruction. *J Am Soc Nephrol* 2005;**16**(12):3623–3630.
63 B. D. Humphreys, M. T. Valerius, A. Kobayashi, *et al.* Intrinsic epithelial cells repair the kidney after injury. *Cell Stem Cell* 2008;**2**(3):284–291.
64 Y. Rinkevich, D. T. Montoro, H. Contreras-Trujillo, *et al. In vivo* clonal analysis reveals lineage-restricted progenitor characteristics in mammalian kidney development, maintenance, and regeneration. *Cell Rep* 2014;**7**(4):1270–1283.
65 R. L. Chevalier, A. Kim, B. A. Thornhill, and J. T. Wolstenholme. Recovery following relief of unilateral ureteral obstruction in the neonatal rat. *Kidney Int* 1999;**55**(3):793–807.
66 M. S. Forbes, B. A. Thornhill, C. I. Galarreta, *et al.* Chronic unilateral ureteral obstruction in the neonatal mouse delays maturation of both kidneys and leads to late formation of atubular glomeruli. *AJP: Renal Physiology* 2013;**305**(12):F1736–F1746.

67 G. Campanholle, G. Ligresti, S. A. Gharib, and J. S. Duffield. Cellular mechanisms of tissue fibrosis. 3. Novel mechanisms of kidney fibrosis. *Am J Physiol Cell Physiol* 2013;**304**(7):C591–C603.

68 E. M. Zeisberg and M. Zeisberg. The role of promoter hypermethylation in fibroblast activation and fibrogenesis. *J Pathol* 2013;**229**(2):264–273.

69 W. Bechtel, S. McGoohan, E. M. Zeisberg, *et al.* Methylation determines fibroblast activation and fibrogenesis in the kidney. *Nat Med* 2010;**16**(5):544–550.

70 S. Meran and R. Steadman. Fibroblasts and myofibroblasts in renal fibrosis. *Int J Exp Pathol* 2011;**92**(3):158–167.

71 J. L. Barnes and Y. Gorin. Myofibroblast differentiation during fibrosis: role of NAD(P)H oxidases. *Kidney Int* 2011;**79**(9):944–956.

72 F. Strutz and M. Zeisberg. Renal fibroblasts and myofibroblasts in chronic kidney disease. *J Am Soc Nephrol* 2006;**17**(11):2992–2998.

73 T. D. Hewitson, W. Y. Ho, and C. S. Samuel. Antifibrotic properties of relaxin: *in vivo* mechanism of action in experimental renal tubulointerstitial fibrosis. *Endocrinology* 2010;**151**(10):4938–4948.

74 M. Zeisberg and E. G. Neilson. Mechanisms of tubulointerstitial fibrosis. *J Am Soc Nephrol* 2010;**21**(11):1819–1834.

75 V. S. LeBleu, G. Taduri, J. O'Connell, *et al.* Origin and function of myofibroblasts in kidney fibrosis. *Nat Med* 2013;**19**(8):1047–1053.

76 R. Kramann, R. K. Schneider, D. P. Dirocco, *et al.* Perivascular Gli1$^+$ progenitors are key contributors to injury-induced organ fibrosis. *Cell Stem Cell* 2015;**16**(1):51–66.

77 B. Rodríguez-Iturbe and G. García García. The role of tubulointerstitial inflammation in the progression of chronic renal failure. *Nephron Clin Pract* 2010;**116**(2):c81–c88.

78 R. L. Chevalier, S. Goyal, and B. A. Thornhill. EGF improves recovery following relief of unilateral ureteral obstruction in the neonatal rat. *J Urol* 1999;**162**(4):1532–1536.

79 J. Li, J. A. Deane, N. V. Campanale, *et al.* Blockade of p38 mitogen-activated protein kinase and TGF-β/Smad signaling pathways rescues bone marrow-derived peritubular capillary endothelial cells in adriamycin-induced nephrosis. *J Am Soc Nephrol* 2006;**17**(10):2799–2811.

80 F. Tögel, K. Weiss, Y. Yang, *et al.* Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol* 2007;**292**(5):F1626–F1635.

81 K. Berger, J.-M. Bangen, L. Hammerich, *et al.* Origin of regenerating tubular cells after acute kidney injury. *Proc Natl Acad Sci U S A* 2014;**111**(4):1533–1538.

82 R. Kramann, M. Tanaka, and B. D. Humphreys. Fluorescence microangiography for quantitative assessment of peritubular capillary changes after AKI in mice. *J Am Soc Nephrol* 2014;**25**(9):1924–1931.

83 A. Advani, K. A. Connelly, D. A. Yuen, *et al.* Fluorescent microangiography is a novel and widely applicable technique for delineating the renal microvasculature. *PLoS ONE* 2011;**6**(10):e24695.

84 C. Xinaris, M. Morigi, V. Benedetti, *et al.* A novel strategy to enhance mesenchymal stem cell migration capacity and promote tissue repair in an injury specific fashion. *Cell Transplant* 2013;**22**(3):423–436.

85 X. Liu, W. Shen, Y. Yang, and G. Liu, Therapeutic implications of mesenchymal stem cells transfected with hepatocyte growth factor transplanted in rat kidney with unilateral ureteral obstruction. *J Pediatr Surg* 2011;**46**(3):537–545.

86 D. Sarkar, P. K. Vemula, W. Zhao, *et al.* Engineered mesenchymal stem cells with self-assembled vesicles for systemic cell targeting. *Biomaterials* 2010;**31**(19):5266–5274.

87 Y. Y. Chen, H. H. Qian, W. W. Zhu, *et al.* Hepatocyte growth factor modification promotes the amelioration effects of human umbilical cord mesenchymal stem cells on rat acute kidney injury. *Stem Cells Dev* 2010;**20**(1):103–113.

88 O. Levy, W. Zhao, L. J. Mortensen, *et al.* mRNA-engineered mesenchymal stem cells for targeted delivery of interleukin-10 to sites of inflammation. *Blood* 2013;**122**(14):e23–e32.

89 F. T. Borges, L. A. Reis, and N. Schor. Extracellular vesicles: structure, function, and potential clinical uses in renal diseases. *Braz J Med Biol Res* 2013;**46**(10):824–830.

90 S. Bruno, B. Bussolati, C. Grange, *et al.* Isolation and characterization of resident mesenchymal stem cells in human glomeruli. *Stem Cells Dev* 2009;**18**(6):867–880.

91 S. Gatti, S. Bruno, M. C. Deregibus, *et al.* Microvesicles derived from human adult mesenchymal stem cells protect against ischaemia–reperfusion-induced acute and chronic kidney injury. *Nephrol Dial Transplant* 2011;**26**(5):1474–1483.

92 V. Cantaluppi, S. Gatti, D. Medica, *et al.* Microvesicles derived from endothelial progenitor cells protect the kidney from ischemia–reperfusion injury by microRNA-dependent reprogramming of resident renal cells. *Kidney Int* 2012;**82**(4):412–427.

93 S. Villanueva, E. Ewertz, F. Carrión, *et al. Mesenchymal stem cell injection ameliorates chronic renal failure in a rat model. Clin Sci (Lond)* 2011;**121**(11):489–499.

94 W. Li, H. Jiang, and J.-M. Feng. Isogenic mesenchymal stem cells transplantation improves a rat model of chronic aristolochic acid nephropathy via upregulation of hepatic growth factor and down-regulation of transforming growth factor β1. *Mol Cell Biochem* 2012;**368**(1):137–145.

95 M. Franquesa, E. Herrero, J. Torras, *et al.* Mesenchymal stem cell therapy prevents interstitial fibrosis and tubular atrophy in a rat kidney allograft model. *Stem Cells Dev* 2012;**21**(17):3125–3135.

96 T. Suzuki, M. Iyoda, T. Shibata, *et al.* Therapeutic effects of human mesenchymal stem cells in Wistar-Kyoto rats with anti-glomerular basement membrane glomerulonephritis. *PLoS ONE* 2013;**8**(6):e67475.

97 S. Villanueva, J. E. Carreño, L. Salazar, *et al.* Human mesenchymal stem cell derived from adipose tissue reduce functional and tissue damage in a rat model of chronic renal failure. *Clin Sci (Lond)* 2013;**125**(4):199–210.

98 K. Cheng, P. Rai, A. Plagov, *et al.* Transplantation of bone marrow-derived MSCs improves cisplatinum-induced renal injury through paracrine mechanisms. *Exp Mol Pathol* 2013;**94**(3):466–473.

99 S. Qi and D. Wu. Bone marrow-derived mesenchymal stem cells protect against cisplatin-induced acute kidney injury in rats by inhibiting cell apoptosis. *Int J Mol Med* 2013;**32**(6):1262–1272.

100 X. Bian, B. Zhang, W. Guo, *et al.* Effects of mesenchymal stem cells transplanted at different time points in a rat remnant kidney model. *Am J Nephrol* 2014;**39**(1):75–84.

101 M. T. Abdel Aziz, M. A. A. Wassef, H. H. Ahmed, *et al.* The role of bone marrow derived-mesenchymal stem cells in attenuation of kidney function in rats with diabetic nephropathy. *Diabetol Metab Syndr* 2014;**6**(1): *34*

102 F. Franchi, K. M. Peterson, R. Xu, *et al.* Mesenchymal stromal cells improve renovascular function in polycystic kidney disease. *Cell Transplant* 2015;**24**:1687–1698.

103 A. Trounson, R. G. Thakar, G. Lomax, and D. Gibbons. Clinical trials for stem cell therapies. *BMC Med* 2011;**9**(1):52.

104 N. Perico, F. Casiraghi, M. Introna, *et al.* Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* 2011;**6**(2):412–422.

105 A. V. Vanikar and H. L. Trivedi. Stem cell transplantation in living donor renal transplantation for minimization of immunosuppression. *Transplantation* 2012;**94**(8):845–850.

106 N. Perico, F. Casiraghi, E. Gotti, *et al.* Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int* 2013;**26**(9):867–878.

107 M. E. J. Reinders, J. W. de Fijter, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med* 2013;**2**(2):107–111.

108 H. L. Trivedi, A. V. Vanikar, V. B. Kute, *et al.* The effect of stem cell transplantation on immunosuppression in living donor renal transplantation: a clinical trial. *Int J Organ Transplant Med* 2013;**4**(4):155–162.

109 Y. Peng, M. Ke, L. Xu, *et al.* Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. *Transplantation* 2013;**95**(1):161–168.

110 A. V. Vanikar, H. L. Trivedi, S. C. Gopal, *et al.* Pre-transplant co-infusion of donor-adipose tissue derived mesenchymal stem cells and hematopoietic stem cells may help in achieving tolerance in living donor renal transplantation. *Ren Fail* 2014;**36**(3):457–460.

111 B. J. Nankivell, R. J. Borrows, C. L.-S. Fung, *et al.* The natural history of chronic allograft nephropathy. *N Engl J Med* 2003;**349**(24):2326–2333.

112 M. E. J. Reinders, M. Roemeling-van Rhijn, M. Khairoun, *et al.* Bone marrow-derived mesenchymal stromal cells from patients with end-stage renal disease are suitable for autologous therapy. *Cytotherapy* 2013;**15**(6):663–672.

113 K. Takahashi, K. Tanabe, M. Ohnuki, *et al.* Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 2007;**131**(5):861–872.

114 M.-H. M. Dahlke. Immunomodulative cell therapy after solid organ transplantation: which cell type when? *Curr Opin Organ Transplant* 2012;**17**(1):33–33

115 L. Lu, J. Q. Zhang, F. J. Ramires, and Y. Sun. Molecular and cellular events at the site of myocardial infarction: from the perspective of rebuilding myocardial tissue. *Biochem Biophys Res Commun* 2004;**320**(3):907–913.

116 A. van Dijk, H. Niessen, W. Ursem, *et al.* Accumulation of fibronectin in the heart after myocardial infarction: a putative stimulator of adhesion and proliferation of adipose-derived stem cells. *Cell Tissue Res* 2008;**332**(2):289–298.

117 L. Y. Eun, H. Song, E. Choi, *et al.* Implanted bone marrow-derived mesenchymal stem cells fail to metabolically stabilize or recover electromechanical function in infarcted hearts. *Tissue Cell* 2011;**43**(4):238–245.

118 A. van Dijk, B. A. Naaijkens, W. J. F. M. Jurgens, *et al.* Reduction of infarct size by intravenous injection of uncultured adipose derived stromal cells in a rat model is dependent on the time point of application. *Stem Cell Res* 2011;**7**(3):219–229.

119 F. L. Hisaw. Experimental relaxation of the pubic ligament of the guinea pig. *Exp Biol Med* 1926;**23**(8):661–663.

120 R. Bathgate, M. L. Halls, E. T. van der Westhuizen, *et al.* Relaxin family peptides and their receptors. *Physiol Rev* 2013;**93**(1):405–480.

121 L. L. Zhao, P. J. P. Roche, J. M. J. Gunnersen, *et al.* Mice without a functional relaxin gene are unable to deliver milk to their pups. *Endocrinology* 1998;**140**(1):445–453.

122 C. S. Samuel, C. Zhao, R. A. D. Bathgate, *et al.* Relaxin deficiency in mice is associated with an age-related progression of pulmonary fibrosis. *FASEB J* 2003;**17**(1):121–123.

123 C. S. Samuel, H. Tian, L. Zhao, and E. P. Amento. Relaxin is a key mediator of prostate growth and male reproductive tract development. *Lab Invest* 2003;**83**(7):1055–1067.

124 X.-J. Du, C. S. Samuel, X.-M. Gao, *et al.* Increased myocardial collagen and ventricular diastolic dysfunction in relaxin deficient mice: a gender-specific phenotype. *Cardiovasc Res* 2003;**57**(2):395–404.

125 C. S. Samuel, C. Zhao, C. P. Bond, *et al.* Relaxin-1-deficient mice develop an age-related progression of renal fibrosis. *Kidney Int* 2004;**65**(6):2054–2064.

126 C. S. Samuel, C. Zhao, R. A. D. Bathgate, *et al.* The relaxin gene-knockout mouse: a model of progressive fibrosis. *Ann N Y Acad Sci* 2005;**1041**:173–181.

127 T. D. Hewitson, I. Mookerjee, R. Masterson, *et al.* Endogenous relaxin is a naturally occurring modulator of experimental renal tubulointerstitial fibrosis. *Endocrinology* 2007;**148**(2):660–669.

128 S. L. Garber, Y. Mirochnik, C. S. Brecklin, *et al.* Relaxin decreases renal interstitial fibrosis and slows progression of renal disease. *Kidney Int* 2001;**59**(3):876–882.

129 S. L. Garber, Y. Mirochnik, C. Brecklin, *et al.* Effect of relaxin in two models of renal mass reduction. *Am J Nephrol* 2003;**23**(1):8–12.

130 E. D. Lekgabe. Relaxin Reverses cardiac and renal fibrosis in spontaneously hypertensive rats. *Hypertension* 2005;**46**(2):412–418.

131 M. Collino, M. Rogazzo, A. Pini, *et al.* Acute treatment with relaxin protects the kidney against ischaemia/reperfusion injury. *J Cell Mol Med* 2013;**17**(11):1494–1505.

132 T. Yoshida, H. Kumagai, T. Kohsaka, and N. Ikegaya. Protective effects of relaxin against cisplatin-induced nephrotoxicity in rats. *Nephron Exp Nephrol* 2014;**128**(1–2):9–20.

133 C. S. Samuel, T. D. Hewitson, E. N. Unemori, and M. L. K. Tang. Drugs of the future: the hormone relaxin. *Cell Mol Life Sci* 2007;**64**(12):1539–1557.

134 M. H. J. Heeg, M. J. Koziolek, R. Vasko, *et al.* The antifibrotic effects of relaxin in human renal fibroblasts are mediated in part by inhibition of the Smad2 pathway. *Kidney Int* 2005;**68**(1):96–109.

135 I. Mookerjee, T. D. Hewitson, M. L. Halls, *et al.* Relaxin inhibits renal myofibroblast differentiation via RXFP1, the nitric oxide pathway, and Smad2. *FASEB J* 2009;**23**(4):1219–1229.

136 B. S. M. Chow, E. G. Y. Chew, C. Zhao, *et al.* Relaxin signals through a RXFP1–pERK–nNOS–NO–cGMP-dependent pathway to up-regulate matrix metalloproteinases: the additional involvement of iNOS. *PLoS ONE* 2012;**7**(8):e42714.

137 A. Jeyabalan, S. G. Shroff, J. Novak, and K. P. Conrad, The vascular actions of relaxin. *Adv Exp Med Biol* 2007;**612**:65–87.

138 A. Bitto, N. Irrera, L. Minutoli, *et al.* Relaxin improves multiple markers of wound healing and ameliorates the disturbed healing pattern of the genetically diabetic mice. *Clin Sci (Lond)* 2013; **125**:575–585.

139 L. Formigli, A.-M. Perna, E. Meacci, *et al.* Paracrine effects of transplanted myoblasts and relaxin on post-infarction heart remodelling. *J Cell Mol Med* 2007;**11**(5):1087–1100.

140 C. S. Samuel, S. Cendrawan, X.-M. Gao, *et al.* Relaxin remodels fibrotic healing following myocardial infarction. *Lab Invest* 2011;**91** (5):675–690.

141 M. M. Bonacchi, S. S. Nistri, C. C. Nanni, *et al.* Functional and histopathological improvement of the post-infarcted rat heart upon myoblast cell grafting and relaxin therapy. *J Cell Mol Med* 2009;**13** (9):3437–3448.

CHAPTER 52

# The biology and potential clinical applications of mesenchymal stromal cells in diseases of the lung

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## 52.1 Introduction to lung disease

### 52.1.1 The global burden of lung disease

Chronic lung disease is responsible for approximately 7% of all deaths worldwide and represents 4% of the global burden of disease [1,2]. This is evidenced by the economic cost of chronic lung diseases in the USA, which is $154 billion in health-care costs each year [3]. Notably, chronic obstructive pulmonary disease (COPD) accounts for most of the disease burden of lung diseases and will be the third leading cause of death worldwide by 2020 [4].

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### 52.1.2 The pathogenesis of lung diseases

The lung has a unique structure of a complex series of branches that end in alveoli (or the gas exchange units). This unique structure makes the lung the largest organ exposed to the environment [5]. This large surface area has allowed the lung in humans to adapt to fight or flight situations by increasing oxygen consumption from 250 to 5500 mL/min and blood flow from 4 to 40 L/min [5]. This significant capacity to adapt has conferred an evolutionary survival advantage for humans and other mammals. We pay a price for the large surface area of the lung, since it is always susceptible to injury.

Inflammation is usually the initial phase in an injury caused by either infective or noninfective causes. A generic insult incurred from cigarette smoke or infection results in infiltration by neutrophils, lymphocytes, and macrophages. Furthermore, injury damages epithelium, endothelium, and the mesenchymal tissue of the lung. Two major processes are

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required for normal repair to occur. These are (1) epithelial and endothelial reconstitution and (2) an intact basement membrane on which the epithelial and endothelial cells can proliferate and repair damaged tissue. However if, there is inadequate reconstitution of epithelium and endothelium a fibro-proliferative phase occurs in which the lung develops scar tissue [6,7]. The fibrotic tissue is composed of fibroblasts, myofibroblasts, and an extracellular matrix comprising predominantly of collagen I.

Chronic tissue-remodeled lung disease is a major cause of morbidity and mortality, and the mechanisms of disease progression remain the subject of intensive research. Factors that have been implicated are increased oxidative stress and cellular senescence. The latter results in impaired telomerase function, resulting in defective repair [8]. In addition, upregulation of adhesion molecules promotes ongoing inflammation.

### 52.1.3 The range of lung diseases

The respiratory tract commences at the nasal passages and ends at the alveoli. The extensive branching of the trachea to bronchioles serves to increase the area for gas exchange and to filter potentially harmful organic and inorganic environmental agents. Thus, anatomically specific conditions such as allergic rhinitis and sinusitis are distinct from bronchitis, bronchiolitis, or diseases affecting the alveoli, such as hypersensitivity pneumonitis. Furthermore, the pleura and mediastinum are additional sites for disease.

The major lung diseases include:

1 Airways disease, such as cystic fibrosis, chronic obstructive pulmonary disease (COPD), bronchiectasis, and asthma.
2 Distal lung disease, such as pneumonia, lung cancer, tuberculosis, acute lung injury (ALI), idiopathic pulmonary fibrosis (IPF), sarcoidosis, interstitial pneumonias, and occupational lung diseases.
3 Airways and distal lung disease, such as lung cancer, tuberculosis, and sarcoidosis.

Unfortunately, the therapeutic options for these diseases are limited to bronchodilators, antimicrobial agents, immunosuppressive agents, and chemotherapeutic agents, as well as lung transplantation for a very small minority. However, these treatments do not adequately address the pathological repair and the progressive tissue remodeling of these disorders. Cell therapies have become an increasing focus of research and can act at several therapeutic levels. Pluripotent cells such as embryonic stem cells and induced pluripotent stem cells can be differentiated *in vitro* into epithelium and endothelium, thereby making them theoretically very useful for the reconstitution of damaged tissue. Multipotent cells such mesenchymal stromal cells (MSCs) can enhance tissue repair and positively immunomodulate pathological inflammatory responses. Cell therapies may provide important treatment options for these lung diseases.

## 52.2 What are stem cells?

Stem cells possess the following characteristics [9]:

1 Derivation from a clonal population and the ability to self-renew, thus creating daughter stem cells.
2 Unlimited doubling capacity.
3 Differentiation into many cell types.

The archetypal human adult stem cell is the hematopoietic stem cell, which gives rise to daughter stem cells and is also capable of differentiating to the cells of the hematopoietic and immune systems.

True mesenchymal *stem* cells have been shown to exist in the bone marrow (see Chapter 32), but are very rare cells; in order to obtain enough mesenchymal cells for laboratory studies, preclinical animal model studies, and clinical trials the undifferentiated progenies of mesenchymal stem cells are produced by *ex vivo* expansion (see Chapters 3, 4, 5 and 7). These cells are most commonly called mesenchymal *stromal* cells, and the abbreviation "MSCs" will be used in this chapter to denote them.

MSCs have anti-inflammatory properties that can reduce inflammatory pathways as well as possibly replacing damaged lung tissue. MSCs derived from different sources [10], including the placenta [11], umbilical cord, and adipose tissue (see Chapters 3–5 and 7), have these anti-inflammatory actions. MSCs are part of the connective tissue framework that provides both structural and physiological support to parenchymal cells in multiple organs. Since they form part of the wall of blood vessels they can be obtained from any vascularized tissue or organ.

## 52.3 What are mesenchymal stromal cells?

MSCs were first isolated by Friedenstein and colleagues in 1966 from suspensions of bone marrow and spleen [12]. According to the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy Standards [9], MSCs have three key characteristics:

1 MSCs are plastic-adherent when cultured under standard conditions.
2 MSCs express the cell surface proteins CD73, CD90, and CD105 and do not express CD45, CD34, CD14, CD11b, CD79α, CD19, or human leukocyte antigen (HLA)-DR surface molecules (see Chapters 1–5 and 7 for further details).
3 MSCs are capable of differentiating into the mesodermal lineages of bone, cartilage, and adipose tissue.

The identification of MSCs is not without difficulty since the listed cell surface markers are ubiquitous and found on dermal fibroblasts [13–15], but see Chapter 30 for features that distinguish MSCs from fibroblasts. MSCs are not a homogeneous cell population since some bone-marrow-derived MSCs (BM-MSCs) may be unipotent, bipotent, or tripotent in terms of their ability to differentiate to osteoblasts, chondrocytes, or adipocytes *in vitro*. This heterogeneity of BM-MSCs likely applies to MSCs derived from other organs such as the lung [16].

## 52.4 Lung-resident mesenchymal stromal cells

MSCs in the lung may provide support for normal epithelial and endothelial homeostasis. Lung MSCs have been isolated from broncho-alveolar lavage fluid and lung tissue [17,18]. McQualter *et al.* demonstrated a stem cell common antigen-$1^+$ population of MSCs in mice responsible for proliferation and differentiation of a bronchiolar epithelial stem/progenitor cell into both airway and alveolar lineages. These actions are mediated by the secretion of fibroblast growth factor [10] and hepatocyte growth factor (HGF) [19]. These studies confirmed that MSCs are central to the maintenance of normal lung function and structure.

## 52.5 Tracking mesenchymal stromal cells in the body

When injected intravenously, many studies have shown that the extensive capillary circulation within the lung acts as a filter for the MSCs and that few of them enter the systemic circulation [20,21]. As an example, intravenously injected indium-labeled MSCs homed to the lung and remained concentrated there for 48 h (Figure 52.1) [22]. The initial signal was strong for the first 20 min, followed by a decline during the subsequent 48 h.

This localization in the lungs is likely due to their large size: their diameter in a single cell suspension is approximately 100 μm, which is considerably greater than the diameter of a pulmonary capillary.

Further studies have suggested that MSCs retained in the lungs appear to target areas of injured lung [23]. Direct intratracheal, intrapulmonary, and intrapleural administration are alternative routes for the administration of MSCs to the lung [23]. As shown in Chapter 22, MSCs are able to pass through the lung barrier and subsequently preferentially home to a site of inflammation – in this case to a small skin incision on one side of the abdomen made just prior to an intravenous injection of murine MSCs. A similar response has been shown for unilateral kidney damage (see Chapter 51).

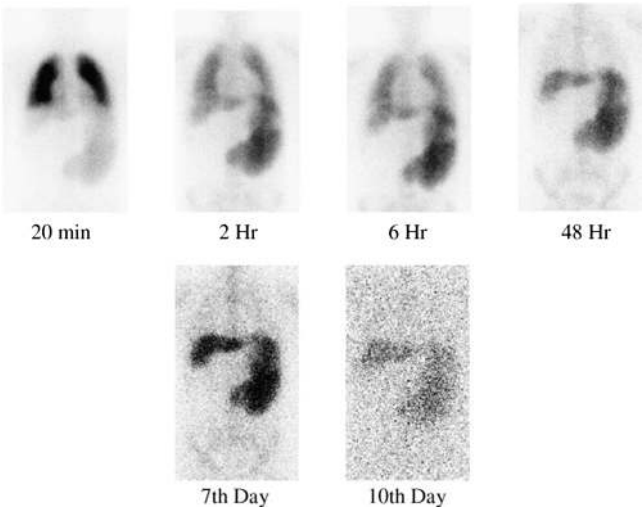



**Figure 52.1** Distribution of indium-labeled MSCs in the lungs, liver, and spleen over 10 days. *Source*: Gholamrezanezhad 2011 [22], pp. 961–967. Reproduced with permission of Elsevier. (See color plate section.)

## 52.6 The properties of mesenchymal stromal cells that favor repair

MSCs have several properties that make them a unique candidate for therapeutic use. They are relatively non-immunogenic, and direct injection into tissues or intravenous or intra-arterial infusion of MSCs in humans has not been associated with adverse events. The effects of MSCs may be dependent on tissue engraftment (although this is controversial), modulation of the immune system, and/or release of trophic (supportive) factors. (See Chapter 32 for an account of their critical role in the survival, growth, and differentiation of hematopoietic stem cells in the bone marrow.)

### 52.6.1 Avoidance of immune recognition

MSCs are able to evade the recipient's immune system (at least to some extent – see Chapter 29), thus enabling the administration of allogeneic MSCs from unrelated HLA-unmatched donors without need for immunosuppression of the recipient. Human MSCs express low levels of major histocompatibility complex (MHC) class I and do not express MHC class II [25]. However, there is a suggestion that MSCs that have differentiated into myocytes may demonstrate upregulation of MHC molecules, leading to their destruction by complement- and/or cell-mediated lysis [26,27]. Furthermore, evidence from a primate model described the development of alloantibodies in two of six animals following multiple administrations of high-dose allogeneic MSCs [28]. In addition, MSCs have also been reported to induce memory T cell responses [29] and activation of the natural killer (NK) cell receptor ligands NKG2D and UL16 [30] in a murine model, thus limiting their ability to avoid lysis by NK cells [31]. Caution must therefore be exercised in future clinical studies that utilize multiple injections of allogeneic MSCs.

### 52.6.2 Mechanisms of mesenchymal-stromal-cell-mediated immunomodulation

MSCs play a major role in immunomodulation and tissue homeostasis. MSCs protect the microenvironment from inflammatory insults by reciprocal regulation of innate and adaptive components of the immune system. These properties have sparked interest in their use as immunosuppressive agents to alleviate chronic inflammatory diseases and graft rejection. The overall regulatory effect of MSCs is a result of many mechanisms that rely on soluble mediators and/or cell-to-cell contact (see Table 52.1).

#### 52.6.2.1 Macrophages

Macrophages are capable of differentiating into pro-inflammatory (M1) and anti-inflammatory (M2) phenotypes. M1

**Table 52.1** Immunomodulatory effects of MSCs.

| Cell type | Mediators | Effects |
|---|---|---|
| *Innate immune system* | | |
| Macrophages | $PGE_2$, IL-6, GM-CSF | ↓ MHC class II, CD86 expression<br>↓ M1 differentiation<br>↑ M2 differentiation<br>↑ IL-10 production |
| Neutrophils | IL-6 | ↓ Oxidative metabolism<br>↓ Apoptosis |
| Mast cells | $PGE_2$, TGF-β1 | ↓ Degranulation<br>↓ Chemotaxis<br>↓ Cytokine production |
| DCs | IL-6, $PGE_2$ | ↓ Maturation<br>↓ Co-stimulatory molecule expression<br>↓ Lymph node migration<br>↓ Endocytosis<br>↑ Regulatory DC generation<br>↑ DC tolerance |
| NK cells | $PGE_2$, TGF-β1, IDO | ↓ Proliferation<br>↓ Cytotoxicity<br>↓ IFN-γ, TNF-α production<br>↓ Activating receptor expression |
| *Adaptive immune system* | | |
| T cells | NO, HGF, PD-1, $PGE_2$, TGF-β1, sHLA-G5 | ↓ Th1 activity<br>↑ Th2 activity<br>↓ Th17 activity<br>↓ $CD8^+$ T cell cytotoxicity<br>↑ IL-10-producing Tregs |
| B cells | PD-1, IDO | ↓ IgG, IgA, and IgM production<br>↓ Plasmablast differentiation<br>↓ Chemokine receptor expression<br>↑ IL-10-producing Bregs |

Breg: regulatory B cell; DC: dendritic cell; GM-CSF: granulocyte-macrophage colony-stimulating factor; IDO: indoleamine 2,3-dioxygenase; IFN: interferon; Ig: immunoglobulin; IL: interleukin; NO: nitric oxide; PD-1: programmed cell death protein 1; $PGE_2$: prostaglandin-$E_2$; TGF: transforming growth factor; Th: T helper; TNF: tumor necrosis factor; Treg: regulatory T cell.

macrophages possess an antimicrobial arsenal that enables them to engulf and destroy invading pathogens, as well the production of an array of proinflammatory cytokines and antimicrobial molecules such as NO. In contrast, M2 macrophages suppress the immune response by producing anti-inflammatory cytokines such as IL-10. MSCs thus favor the activity of M2 macrophages while antagonizing M1 activity. MSCs also downregulate the expression of CD86 and MHC class II on macrophages to reduce their responsiveness to inflammatory stimuli [32]. These effects been shown to occur in a process dependent on prostaglandin-$E_2$ ($PGE_2$), which is constitutively produced by MSCs but can be upregulated in response to proinflammatory cytokines. Moreover, MSC-derived IL-6 and granulocyte-macrophage colony-stimulating factor can synergistically favor the polarization of M2 macrophages. Further information on the interactions between MSCs in the innate and immune systems are given in Chapters 33 and 34.

#### 52.6.2.2 Neutrophils

Neutrophils play an important role in the first line of defense against pathogens. Neutrophils release an array of reactive oxygen species in a process called respiratory burst which is used as a defense mechanism against invading microbes. Accumulation of reactive oxygen species is highly inflammatory and is associated with the rapid apoptosis of neutrophils. In an IL-6-dependent manner, MSCs inhibit respiratory burst and rescue neutrophils from apoptosis. In contrast, the phagocytic capacity, expression of adhesion molecules, and chemotaxis of neutrophils in response to IL-8 are not affected, suggesting that MSCs may act to preserve critical neutrophil functions while preventing

excessive oxidative burst and subsequent inflammation or tissue damage [33].

#### 52.6.2.3 Mast cells

Mast cells play a critical role in allergic responses and anaphylaxis through the release of proinflammatory cytokines and the degranulation of histamine. After interacting with MSCs mast cell functions, including chemotaxis, degranulation, and cytokine production are severely inhibited. These processes were found to be dependent on MSC-derived $PGE_2$ [34]. Moreover, in response to IL-4, TGF-β1 produced by MSCs contributes to the attenuation of mast cell degranulation by reducing FcεRI expression [35]. The high-affinity receptor FcεRI is a member of the Ig superfamily. It has two Ig-like domains. FcεRI is found on epidermal Langerhans cells, eosinophils, mast cells, and basophils. As a result of its cellular distribution, it plays a major role in controlling allergic responses. FcεRI is also expressed on antigen-presenting cells and controls the production of cytokines that mediate immune reactions that promote inflammation. (ε is the Greek symbol epsilon and is the fifth letter in the Greek alphabet.)

#### 52.6.2.4 Natural killer cells

NK cells are cytotoxic cells of the innate immune system that are pivotal to immune surveillance against intracellular pathogens and malignant cells. The cytotoxic effects of NK cells are regulated by both activating and inhibiting receptors but can also be activated following antibody recognition during antibody-dependent cellular toxicity. Following stimulation by MSC-derived $PGE_2$ and TGF-β1, NK cell cytoxicity and proliferation were inhibited. Moreover, expression of proinflammatory cytokines, IFN-γ, and TNF-α and activation of the $PGE_2$ receptor 4 (EP4) and NKG2D were reduced [31]. (NKG2D is a transmembrane protein belonging to the CD94/NKG2 family of C-type lectin-like receptors. In humans it is expressed by NK cells, γδ T cells, and $CD8^+$ αβ T cells. NKG2D is an activating/costimulatory receptor.)

The activity of IDO in MSCs may also operate synergistically with $PGE_2$ to inhibit NK cell function [36]. IDO is an enzyme that produces the immune regulatory catabolite tryptophan. Although IDO is not the mediator, immune regulation via this pathway is dependent on IDO activity. Some mechanisms have been proposed but remain uncharacterized.

#### 52.6.2.5 Dendritic cells

Dendritic cells (DC) are classified as the interface between the innate and the adaptive immune systems by capturing, processing, and presenting antigens to T cells (see Chapters 33 and 34). DC activity is pivotal to the polarization of T cell responses. During DC maturation there is an upregulation of several signals that augment T cell priming. Culturing MSCs with DCs showed reduction of the activating potential of DCs by diminishing their endocytic activity, migration to lymph nodes, and expression of costimulatory molecules (CD40, CD80, CD83, and CD86) following stimulation [37]. Soluble factors produced by MSCs have also been shown to inhibit monocyte differentiation to DCs. Furthermore, the presence of MSCs skews DC differentiation to a regulatory phenotype to promote the expansion of Tregs, thus contributing to the state of immunosuppression. These effects are largely attributed to the action of IL-6 and $PGE_2$, but unknown contact-dependent mechanisms have also been suggested [38,39]. Overall, MSCs promote DC tolerance by attenuating their ability to prime the adaptive arm of the immune response.

#### 52.6.2.6 B cells

B cells play a protective role against pathogens by producing highly specific antibodies that serve to neutralize pathogens as well as to enhance effector cell functions. *In vitro* studies have demonstrated that MSCs inhibit B cell proliferation by cell cycle arrest [40]. In addition, MSCs downmodulate IgM, IgG, and IgA production and chemokine receptor expression [41]. A contact-dependent mechanism involving PD-1 (also known as CD279) has been implicated in this process. However, soluble factors also play a role [42]. More recently, the discovery of a new Breg subset has highlighted novel functions for B cells. Following MSC infusion the proportion of IL-10-producing Bregs was increased in a model for graft-versus-host disease. This was at least partially dependent on IDO expression [43].

#### 52.6.2.7 T cells

T cells are classified into several subsets that can directly kill pathogens (cytotoxic T lymphocytes (CTLs)) or help coordinate immune responses (T helper (Th)1, Th2, or Th17 cells). MSCs strongly inhibited T cell proliferation, which required MSC-derived HGF, TGF-β1, and NO produced by inducible NO synthase [44,45]. This effect was also exacerbated by the action of MSCs on other immune cells, including DCs for which T cell activation is dependent. In contrast to the effects on cytotoxic and Th cell subsets, MSCs increased the proportion of IL-10 producing Tregs, which are an immunosuppressive T-cell subset. MSC blocking studies confirmed soluble HLA G5 (sHLA-G5) to be a mediator of this effect. However, contact between cells was required to maximize the production of sHLA-G5 [46].

MSCs promote IL-4-producing Th2 cells, which exert an anti-inflammatory effect and inhibit IFN-γ-producing Th1 cells, which are generally considered proinflammatory. $PGE_2$ appears to be one of the strongest mediators for regulating this balance [47], although several other soluble factors, including IDO, may have additive effects [48]. Furthermore, Th17 development and subsequent IL-17 production are also markedly impaired; this is also dependent on $PGE_2$ production, PD-1/PD-L1 ligation, and other cell-to-cell contacts by MSCs [49,50]. Finally, MSCs also impair the activity of CTLs. *In vitro* studies have shown that co-culture with MSCs reduced the expression of IFN-γ, CD8, and granzyme-B production in CTLs, which markedly inhibited their cytotoxicity. It has been suggested that the inhibition of CTL activity is indirectly a result of diminished DC activity [51].

### 52.6.3 Mesenchymal-stromal-cell-mediated repair via trophic factors

There has been ongoing controversy as to whether MSCs engraft or release trophic factors that mediate their effects. Early studies demonstrated differentiation into mesodermal-derived tissue such as adipose and bone and muscle. In addition, there were reports that MSCs also differentiated into non-mesodermal-derived tissue, including bronchial epithelium, renal epithelium, neuronal tissue, and cardiomyocytes [52]. However, it is becoming increasingly evident that the effect of MSCs is not dependent on engraftment. The paradigm change was stimulated by a study of MSCs in people with osteogenesis imperfecta (brittle bone disease), a congenital disease of defective connective tissue usually because of a deficiency of type I collagen (see Chapter 47). In this study there was a very low level of engraftment in the bone marrow (<1%), while significant functional improvements were noted [53]. There are also studies that demonstrate a treatment effect without significant engraftment in other organs, including the brain [54], heart [55], and kidney [56].

#### 52.6.3.1 Growth factors

Several studies have shown that growth factors have a role in mediating repair. In an innovative explant (perfused) lung model, Lee *et al.* subjected the lung to lipopolysaccharide (LPS, also known as endotoxin) injury that increased alveolar permeability. MSCs inhibited the increase in capillary permeability. These researchers knocked-down several growth factors, including HGF, fibroblast growth factor, and keratinocyte growth factor (KGF), but found that only KGF knockdown inhibited the actions of MSCs [57]. Although HGF has not been noted to have mediated repair in the lungs, it has been found to mediate improvement in a model of acute kidney damage [58].

#### 52.6.3.2 Microvesicles

A compelling development in MSC research is the discovery that MSCs release microvesicles (MVs) (see Chapters 1, 10, 20 and 38). These MVs are biologically active and appear to mediate repair as effectively as MSCs themselves. MVs are nuclear plasma-membrane-bound circular fragments of 50–200 nm in size that are constitutively released from the endosomal compartment as exosomes or shed from the plasma membrane [38,59]. The MVs derived from MSCs also appear to be as immune-privileged MSCs themselves, since they express very low levels of MHC class I or class II antigens. A wide variety of molecules are carried by MVs, including microRNA (miRNA), mRNA, and proteins.

Studies have shown that MSC-derived MVs augmented repair in a murine glycerol-induced model of acute kidney injury by increasing the proliferation of renal tubular cells [60]. The hypothesized mechanism is that the MSC MVs homed to, and were incorporated into, the injured tubular cells via the surface receptor CD44 (the extracellular matrix receptor type III for hyaluronic acid), thus allowing for transfer of MSC MVs' mRNA. Another study found that MSC MVs attenuated pulmonary edema and lung protein permeability in an *Escherichia coli* endotoxin-induced ALI in mice through the expression of KGF mRNA in the injured alveoli [61]. In another study, Gennai *et al.* demonstrated that MSC MVs improved alveolar fluid clearance in an *ex vivo* perfusion model [62]. The administration of intravenous MSC MVs improved lung mechanics and induced a reduction of pulmonary artery pressure that was probably due to an increase in NO. The internalization of MVs into injured cells was mediated through the CD44 receptor [62]. This is further evidence that MSC MVs are a potentially important new tool in the treatment of lung diseases.

#### 52.6.3.3 Mitochondria

Another proposed mechanism for the reparative effects of MSCs is the transfer of normal MSC mitochondria to injured cells. In a seminal study, Islam *et al.* demonstrated that mitochondrial transfer rescued injured cells by improving their energetics [63] (see Chapters 10 and 21 for further details).

#### 52.6.3.4 mRNA and microRNA

The contents of MSC MVs extend to miRNA. Eirin *et al.* documented the contents of extracellular MVs derived from adipose-tissue-derived MSCs. MVs preferentially expressed mRNA for transcription factors such as MyoD family inhibitor domain containing (MDFIC), POU domain, class 3, transcription factor 1 (POU3F1), and nuclear receptor-interacting protein 1 (NRIP1), as well as genes involved in angiogenesis, such as HGF, transcription factor HES1 (hairy and enhancer of split-1) and TCF4 (sometimes referred to as immunoglobulin transcription factor 2TCF4). In addition, mRNA for genes involved in adipogenesis, such as CCAAT/enhancer binding protein alpha and Kruppel-like factor 7, were preferentially expressed in these MVs. In addition, they also carried miRNAs miR148a, miR532-5p, miR378, and let-7f. This study demonstrated that MSC MVs can carry miRNA with the potential to modify recipient tissue function [64].

In a mouse model of hypoxia-induced pulmonary hypertension, MVs downregulated miR-17 and upregulated miR-204. miR-17 is associated with signal transducer and activator of transcription 3 signaling and is upregulated in pulmonary hypertension [65]. MiR-204 is downregulated in IPF with pulmonary hypertension [66]. The change in the profile of miRNA following MV injection supports the potential therapeutic role of MVs in therapy.

## 52.7 Mesenchymal stromal cells as delivery agents for drugs

MSCs not only are used as direct therapy for lung disorders but may also be used as engineered vectors for drug delivery to specific sites (see Chapters 61 and 62).

### 52.7.1 Viral transduction

In a study by Sage *et al.* MSCs were transduced using a lentiviral vector to express TNF-related apoptosis-inducing ligand

(TRAIL) [67]. TRAIL is a proapoptotic molecule for cancer cells but does not affect healthy cells. These cells were used to treat pleural mesothelioma in mice. MSCs were delivered directly into the tumor or systemically in a mouse model of mesothelioma. Intravenously administered MSCs resulted in increased engraftment and tumor reduction due to elevated apoptosis. Of note was that intrapleural instillation of MSCs were not as effective [67]. This study elegantly illustrated that MSCs can be engineered to express therapeutic molecules for the treatment of specific diseases.

### 52.7.2 Genetic modulation

Several strategies to modify MSC protein expression include genetic modulation by viral and nonviral transduction. Viral transduction is potentially dangerous in human subjects. Therefore, nonviral strategies designed to increase the permeability of the cell membrane and nucleus have been utilized to improve transfection, including electroporation. Such techniques have been used to increase MSC survival by expression of the anti-apoptotic molecule Bcl-2, improve homing of cells to sites of injury via C–X–C motif chemokine ligand 4 and to deliver bone-forming molecules such as bone morphogenetic protein 2 and TGF-β to facilitate bone repair [68].

### 52.7.3 Nanoparticle incorporation

In addition to genetic manipulation, nanoparticles can be incorporated into MSCs to enable the expression of specific molecules via cellular internalization. Nanoparticles can be endocytosed. In addition, nanoparticles can be incorporated into cells by lipid fusion. Nanoparticles are taken up by the cell and released through endocytosis by fusion with the cell membrane [68].

### 52.7.4 Surface modification

The homing of MSCs can be enhanced by modifying their cell surface characteristics. Since selectins are upregulated on the endothelium of injured tissue, Xia *et al.* enzymatically modified umbilical cord blood cells to express selectin binding motifs and increased MSC delivery to sites of injury [69]. Increased delivery of MSCs can also be achieved through chemical modification by covalently coupling target ligands to the cell surface [70]. Another strategy is to chemically paint MSCs with palmitated-ischemia homing particles that also improve MSC trafficking to injured tissue [71].

### 52.7.5 Preconditioned mesenchymal stromal cells

MSCs can be further manipulated for their application in lung diseases. Lan *et al.* demonstrated that exposing MSCs to a hypoxic environment improved repair in a bleomycin-induced model of lung injury. The hypoxia exposure improved the homing characteristics of MSCs [72]. It is possible that MSCs alone may be surpassed by engineered MSCs for a variety of specific tasks in the treatment of cancer and tissue injury.

## 52.8 Preclinical and clinical studies of mesenchymal stromal cells in pulmonary diseases

MSCs have been the focus of a significant number of preclinical trials. Several different animal models have been used to test the efficacy and mechanisms of these cells. In this chapter we have limited our discussion to IPF, COPD, and acute respiratory distress syndrome (ARDS). However, we have included a summary of selected preclinical studies of pulmonary hypertension (Table 52.2), asthma (Table 52.3) and bronchopulmonary dysplasia (Table 52.4).

### 52.8.1 Idiopathic pulmonary fibrosis

#### 52.8.1.1 Epidemiology of idiopathic pulmonary fibrosis

Lung fibrosis is a term that applies to scarring of the lung. IPF is a severe and progressive lung disorder resulting from damage to

**Table 52.2** Therapeutic effects of MSC infusion in hypoxia or monocrotaline-induced pulmonary arterial hypertension.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↑ Survival | Sprague-Dawley rat | Sprague-Dawley rat | [73–75] |
| ↑ Hemodynamics | Sprague-Dawley rat | BM – Sprague Dawley rat | [75] |
| | Lewis rat | BM – Lewis rat | [76] |
| ↓ Pulmonary arterial pressure | Sprague-Dawley rat | BM – Dawley rat | [74,77] |
| | Wistar rat | BM – Wistar rat | [78] |
| ↓ Right ventricular systolic pressure | Sprague-Dawley rat | BM – Sprague Dawley rat | [73] |
| | Lewis rat | BM – Lewis rat | [79] |
| | FVB/n mouse | BM – FVB/n mouse | [80] |
| ↓ Right ventricular hypertrophy | Wistar rat | BM – Wistar rat | [78] |
| | FVB/n mouse | BM – FVB/n mouse | [80] |

BM: bone marrow.

**Table 52.3** Therapeutic effects of MSC infusion in preclinical models of asthma.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↓ Eosinophils, macrophages, neutrophils or mast cells | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – BALB/c mouse | [82,83] |
| | BALB/c mouse | BM – Sprague-Dawley rat | [82,84] |
| | C57BL/6 J mouse | BM – C57BL/6 J mouse | [85] |
| ↓ Goblet cells | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – BALB/c mouse | [83] |
| ↓ Epithelial thickening | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – BALB/c mouse | [83] |
| | C57/BL/6 | BM – C57BL/6 mouse | [84] |
| ↓ Collagen deposition | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – Sprague-Dawley rat | [85] |
| ↓ Serum IgE | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – BALB/c mouse | [82] |
| | C57BL/6 J mouse | BM – C57BL/6 J mouse | [82] |
| ↓ Serum nitric oxide | BALB/c mouse | BM – BALB/c mouse | [83] |
| ↓ Th2 cytokines (e.g., IL-13) | BALB/c mouse | BM – human | [81] |
| | BALB/c mouse | BM – BALB/c mouse | [82] |
| | C57BL/6 J mouse | BM – C57BL/6 J mouse | [82] |
| ↑ Anti-inflammatory cytokines (e.g., TGF-β) | BALB/c mouse | BM – BALB/c mouse | [82] |
| | C57BL/6 J mouse | BM – C57BL/6 J mouse | [82] |

BM: bone marrow.

**Table 52.4** Effects of MSC infusion in hyperoxia-induced bronchopulmonary dysplasia.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↑ Survival | Sprague-Dawley rat | BM – Sprague-Dawley rat | [86] |
| | Kunming mouse | BM – Kunming mouse | [87] |
| ↑ Lung function | Sprague-Dawley rat | BM – Sprague-Dawley rat | [86] |
| | FVB mouse | BM – FVB mouse | [88,89] |
| ↓ Alveolar loss/injury and improved lung architecture | Sprague-Dawley rat | BM – Sprague-Dawley rat | [86,90] |
| | FVB mouse | BM – FVB mouse | [88,91] |
| | Kunming mouse | BM-Kunming mouse | [87] |
| ↓ Inflammation | FVB mouse | BM – FVB mouse | [91] |
| ↓ Pulmonary arterial hypertension | Sprague-Dawley rat | BM – Sprague-Dawley rat | [86,90] |
| | FVB mouse | BM – FVB mouse | [88,91] |
| ↓ Vessel remodeling | Sprague-Dawley rat | BM – Sprague-Dawley rat | [86] |
| | FVB mouse | BM – FVB mouse | [88,91] |
| ↓ Fibrosis and profibrotic cytokines | Kunming mouse | BM – Kunming mouse | [87] |

BM: bone marrow.

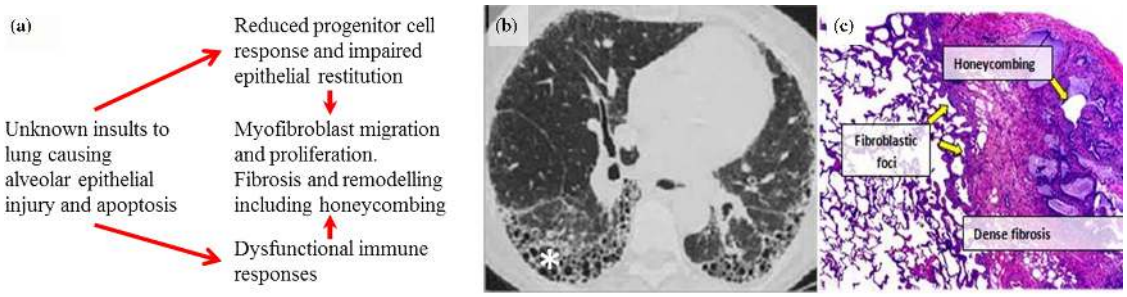



**Figure 52.2** (a) Pathogenesis of IPF. (b) High-resolution computed tomogram of IPF with honeycombing (*). (c) Histopathology of IPF. (See color plate section.)

the lung tissue by inflammation and fibrosis with a median survival from diagnosis of 2–5 years [92]. IPF has an incidence ranging from 4/100 000 in persons aged 18–34 years to >200/100 000 among those above 75 years of age [93].

#### 52.8.1.2 Pathogenesis of idiopathic pulmonary fibrosis

The pathogenesis of IPF (Figure 52.2b) demonstrates the cycle of injury and remodeling of the distal lung. The process results in the characteristic radiology of bibasal honeycombing, reticular distortion, and traction bronchiectasis (Figure 52.2b) [94]. The histopathology of IPF typically shows the presence of fibroblastic foci, fibrosis and honeycombing (Figure 52.2c) [92]. Chronic remodeling of the lung results in the loss of gas-exchange units and the clinical features of IPF.

#### 52.8.1.3 Limitations of the preclinical model of idiopathic pulmonary fibrosis using bleomycin

Animal models have been predominantly used in bleomycin-induced lung injury (Table 52.5). This model has its limitations when compared with human IPF. The bleomycin-induced murine model is characterized by inflammation and fibrosis. In contrast, inflammation is not a major feature of IPF. Furthermore, the bleomycin model is a transient disease model with recovery over 6–8 weeks, and tissue remodeling such as honeycomb formation is not significant. Thus, the preclinical studies in mice may not reflect the outcomes for people with IPF. The timing of MSC administration in the bleomycin mouse model is important. MSCs administered within 24 h of bleomycin injury attenuated inflammation and fibrosis. MSCs administered 7 days after bleomycin injury did not attenuate fibrosis. This finding suggests that MSCs reduce inflammation and the downstream effects of fibrosis as opposed to having a direct antifibrotic effect. This was confirmed in a study by our group comparing the effects of several types of adult stem cells in which MSCs did not have a major impact on reducing established fibrosis.

#### 52.8.1.4 The safety of mesenchymal stromal cells in a clinical trial in patients with idiopathic pulmonary fibrosis

Human studies using MSCs in IPF have been performed. Chambers *et al.* demonstrated that placenta-derived MSCs were safe to administer systemically in IPF patients, although at 6 months the forced vital capacity, diffusing capacity for carbon monoxide, 6 min walk distance, and computed tomography fibrosis score were unchanged compared with baseline. There was no evidence of worsening fibrosis [99].

**Table 52.5** Therapeutic effects of MSC infusion on bleomycin-induced lung fibrosis.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↓ Hydroxyproline – a quantitative measure of tissue fibrosis and collagen deposition | C57BL/6 mice | BM – BALB/c mice | [95] |
| | Sprague-Dawley rat | BM – Sprague-Dawley rat | [96,97] |
| | SCID mice | UC – human | [10] |
| ↓ Lung fibrotic score | Sprague-Dawley rat | BM – Sprague-Dawley rat | [96] |
| | C57BL/6 mice | Endogenous recruitment using hyaluronidase | [98] |
| ↓ Collagen | SCID mice | UC – human | [10] |
| | C57BL/6 mice | Promote endogenous recruitment using hyaluronidase | [98] |
| ↓ Profibrotic cytokines | Sprague-Dawley rat | BM – Sprague-Dawley rat | [96] |
| | C57BL/6 mice | Endogenous recruitment using hyaluronidase | [98] |

BM: bone marrow; UC: umbilical cord.

## 52.8.2 Chronic obstructive pulmonary disease

### 52.8.2.1 Chronic bronchitis and emphysema

COPD is the commonest respiratory condition in the world. It ranks amongst the top five causes of global morbidity and mortality, with 1.2 million patients in Australia [100]. COPD has two broad definitions that incorporate characteristics of the clinical features.

Chronic bronchitis is defined as daily sputum production for three consecutive months for two consecutive years and involves damage to the airways and the chronic production of mucus.

Emphysema is a radiological definition in which there is dilatation of the distal air spaces beyond the terminal bronchi. The damage to the distal lung in COPD is due to loss of elastin. These well-established definitions, however, do not take into account the complexity of COPD in which some patients deteriorate, lose weight, or have frequent acute exacerbations. A consensus statement released by the Global Initiative for Chronic Obstructive Lung Disease based on symptoms and lung function tests has been published [101].

### 52.8.2.2 Pathogenesis of chronic obstructive pulmonary disease

COPD is characterized by chronic irreversible inflammation and destruction of lung tissue leading to airflow obstruction and loss of gas-exchange units [102]. Smoking is the major risk factor for COPD, resulting in damage to the epithelium and endothelium in the lungs. There is increased oxidant and inflammatory damage resulting in infiltration and activation of immune cells, including neutrophils, macrophages, and $CD4^+$ and $CD8^+$ T cells in the airways of COPD patients [102]. Cell infiltration is driven by cytokines, chemokines, and growth factors. Other pathogenic features of COPD include the imbalance between matrix metalloproteinases (MMPs) and tissue inhibitors of MMPs. The increase in MMPs causes breakdown of the extracellular matrix in the lung, especially of elastin. This causes gas trapping, resulting in physical discomfort. In addition, there is increased apoptosis of alveolar epithelial cells that results in loss of gas-exchange units. The progressive inflammation in the lung also induces a heavy oxidant burden, resulting in cell death. COPD-associated injuries are not limited to the lung but also result in systemic inflammation with an increased incidence of ischemic heart disease, vascular disease, muscle wasting, and cachexia [102].

### 52.8.2.3 A preclinical model of chronic obstructive pulmonary disease: the use of mesenchymal stromal cells in smoke and papain-induced emphysema

Several studies in animal models have demonstrated that MSCs may address the fundamental defects in COPD (Table 52.6).

MSC infusion in a rat model of smoking-induced emphysema reduced proinflammatory cytokines including IL-1β, IL-6, TNF-α and MCP-1 and proteases including MMP-9 and MMP-12, but increased anti-inflammatory cytokines such as TGF-β. In addition MSCs also decreased alveolar epithelial apoptosis. Mechanisms for this inhibition of apoptosis were likely down-regulation of Bax and upregulation of Bcl-2 in a papain-induced emphysema model [104]. MSCs reduce cleavage of caspase-3 and increase VEGF, which also inhibit cell apoptosis [103].

COPD is also characterized by an increase in the oxidant : antioxidant level induced by the transcription factors nuclear factor kappa light chain-enhancer of activated B cells and activator protein 1. The increase in oxidant levels in COPD results in increased inflammation, DNA damage, MMP levels, and cell apoptosis [107,108]. MSCs have been shown to decrease oxidant stress in an animal model [109,110]. Mechanisms include decreasing malondialdehyde and increasing heme oxygenase-1 levels in the lung with consequent cytoprotective effects. Thus, preclinical studies have shown that MSCs can improve pathogenic outcomes in COPD by addressing specific events in the course of the disease.

**Table 52.6** Therapeutic effects of MSC infusion on smoke- and papain-induced emphysema.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↑ Lung function | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103] |
| ↓ Emphysema | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103] |
| | Lewis rat | BM – Lewis rats | [104,105] |
| ↓ Proinflammatory cytokines (e.g., IL-6) | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103] |
| ↓ Proteases (e.g., MMP) | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103] |
| ↓ Apoptosis | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103,109] |
| | Lewis rat | BM – Lewis rat | [104,105] |
| ↑ VEGF | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103,106] |
| ↑ Anti-inflammatory cytokines (e.g., TGF-β) | Sprague-Dawley rat | BM – Sprague-Dawley rat | [103] |

BM: bone marrow.

#### 52.8.2.4 Safety of mesenchymal stromal cells in chronic obstructive pulmonary disease

Weiss *et al.* carried out studies on the safety of human MSCs in COPD patients [111]. Sixty-two patients were randomized to double-blinded intravenous infusions of either allogeneic MSCs or vehicle control. Patients received four monthly infusions ($100 \times 10^6$ cells/infusion) and were followed up for 2 years. All patients completed the trial protocol, and 74% completed the 2-year follow-up. There were no toxicity or adverse side effects noted. There was no difference in the frequency of COPD exacerbations or worsening of disease in patients treated with MSCs versus controls. There was also no difference in pulmonary function tests or quality-of-life indicators between MSC-treated patients and control patients. Interestingly, there was a drop in the inflammatory marker C-reactive protein in MSC-treated patients who had elevated C-reactive protein levels at entry of the study. The authors concluded that intravenous administration of MSCs was safe in patients with moderate to severe COPD [111]. There are several ongoing studies delineating a potential role of MSCs as a treatment for COPD.

### 52.8.3 Acute lung injury and acute respiratory distress syndrome

#### 52.8.3.1 Epidemiology of acute lung injury/acute respiratory distress syndrome

ALI and the more severe ARDS are the commonest cause of acute respiratory failure in hospitalized patients. ALI is defined as the ratio of arterial oxygen partial pressure to fractional inspired oxygen ($PaO_2 : FiO_2$) of <300, while the more severe ARDS is defined as a ratio of <200. ARDS is characterized by refractory hypoxemia in patients with bilateral lung infiltrates in the absence of cardiogenic pulmonary oedema [112,113]. A National Institutes of Health study estimated the incidence of ARDS to be 75 per 100 000 population in the USA with a mortality rate of 40–70% [114].

#### 52.8.3.2 Pathogenesis of acute lung injury/acute respiratory distress syndrome

ARDS can be caused by direct or indirect injuries to the lung, including aspiration of gastric contents and sepsis [115]. The early phase of lung injury is characterized by epithelial and endothelial cell damage leading to a compromised alveolar–capillary barrier and exudation of fluid into the alveolar space, followed by infiltration of inflammatory cells such as neutrophils. Progression from acute lung injury to fibrosis is observed 5–7 days after the injury [116], and this phase predicts a poor prognosis [117]. Current therapies, such as lung-protective ventilation and a fluid-conservative strategy, although useful, are of limited efficacy [118,119]. Thus, innovative therapies are needed to address the fundamental defects in ARDS. Human MSCs exhibit anti-inflammatory and proreparative properties that may be beneficial in ARDS.

#### 52.8.3.3 Preclinical model of acute lung injury/acute respiratory distress syndrome by endotoxin- or bleomycin-induced lung injury

To date MSCs have been studied extensively in preclinical lung injury models (Table 52.7) [118,130]. Most of the injuries involved intratracheal endotoxin exposure or bleomycin administration, and rodents were the main small animals studied. There have also been studies in larger animal models. In the sheep model of bacterial pneumonia Asmussen *et al.* used a cotton smoke inhalation model and infection with pseudomonas to determine if human MSCs could improve this sheep model of pneumonia [122]. At 24 h after the administration of human MSCs pulmonary artery pressures were reduced and the $PaO_2/FiO_2$ ratio was increased. There were no differences in airway pressure between control sheep and MSC-treated sheep. There were also no difference in any physiological parameters between controls and MSC-treated groups at time points before 24 h [122].

ARDS is also characterized by decreased alveolar fluid clearance due to increased epithelial permeability and high levels of proinflammatory molecules, which allows the influx of protein-rich fluid into the alveoli. MSCs secrete KGF, an epithelial growth factor that increases alveolar clearance by upregulating α-epithelial sodium channel gene expression and Na/K-ATPase activity or by enabling increased trafficking of sodium transport proteins to the cell surface [57,131–133]. Factors such as angiopoietin-1, a ligand for the endothelial Tie2 receptor, increase endothelial stabilization, thus reducing endothelial permeability. Angiopoietin-1 also inhibits leukocyte–endothelium interactions by modifying cell adhesion molecules and cell junctions [134,135].

Specific actions of MSCs in relation to ARDS that could modify the disease include immunomodulation: the anti-inflammatory stimulation of T regulatory lymphocytes (Tregs) and the increase of M2 macrophages could reduce the intense inflammation seen in ARDS. Sun *et al.* demonstrated prevention of ALI by MSC administration, and this correlated with increased levels of anti-inflammatory Tregs and the anti-inflammatory cytokine IL-10 while reducing levels of proinflammatory cytokines such as IFN-γ and TNF-α [129].

MSCs also secrete antimicrobial factors that could improve the outcome of sepsis as a cause for ARDS. MSCs have toll-like receptors and formyl peptide-like receptors that are activated in response to bacterial products [24,136]. MSCs can inhibit bacterial growth by secretion of antimicrobial peptides such as LL-37 [128] and antibacterial proteins such as lipocalin-2 [120]. These improved bacterial clearance in a mouse model of *Escherichia coli* pneumonia. MSCs can also improve bacterial clearance by enhanced phagocytic activity [137].

**Table 52.7** Therapeutic effects of MSC infusion in preclinical models of ALI.

| Beneficial effects | Species | MSC source | Reference |
|---|---|---|---|
| ↑ Survival | C57BL/6 mouse | BM – C57BL/6 mouse | [24,120] |
| | ICR mouse | UC – human | [121] |
| ↑ $PaO_2/FiO_2$ ratio | Sheep | BM – human | [122] |
| ↓ Total protein in BAL | C57BL/6 mouse | BM – C57BL/6 mouse | [24] |
| | Wistar rat | BM – Wistar rat | [123] |
| | Kunming mouse | BM – Kunming mouse | [124] |
| ↓ Pulmonary permeability and edema | C57BL/6 mouse | BM – C57BL/6 mouse | [24,120,125–127] |
| | ICR mouse | UC – human | [121] |
| | Sheep | BM – human | [122] |
| ↓ Interalveolar septal thickening | C57BL/6 mouse | BM – C57BL/6 mouse | [125,126] |
| ↓ Neutrophilia or myeloperoxidase activity | C57BL/6 mouse | BM – C57BL/6 mouse | [125–127] |
| | C57BL/6 mouse | BM – human | [128] |
| | Wistar rat | BM – Wistar rat | [123] |
| | Kunming mouse | BM – Kunming mouse | [124] |
| | ICR mouse | UC – human | [121] |
| ↓ Apoptosis | C57BL/6 mouse | BM – C57BL/6 mouse | [125] |
| ↓ Proinflammatory cytokines (e.g., TNF-α) | C57BL/6 mouse | BM – C57BL/6 mouse | [24,125,126] |
| | C57BL/6 mouse | BM – human | [128] |
| | ICR mouse | UC – human | [121] |
| | Wistar rat | BM – Wistar rat | [123] |
| | Kunming mouse | BM – Kunming mouse | [124] |
| | BALB/c mouse | UC – human | [129] |
| ↑ Anti-inflammatory cytokines (e.g., IL-10) | C57BL/6 mouse | BM – C57BL/6 mouse | [24,127] |
| | Wistar rat | BM – Wistar rat | [123] |
| | Kunming mouse | BM – Kunming mouse | [124] |
| | BALB/c mouse | UC – human | [129] |
| ↑ Tregs | BALB/c mouse | UC – human | [129] |
| ↓ Bacterial load | C57BL/6 mouse | BM – C57BL/6 mouse | [120] |
| | C57BL/6 mouse | BM – human | [128] |
| | ICR mouse | UC – human | [121] |

BAL: bronchoalveolar lavage; BM: bone marrow; $PaO_2/FiO_2$: ratio of partial pressure arterial oxygen and fraction of inspired oxygen; UC: umbilical cord.

#### 52.8.3.4 Demonstration of the potential clinical use of mesenchymal stromal cells in an *ex vivo* human lung model of acute respiratory distress syndrome/acute lung injury

An *ex vivo* perfused human lung model has been used to assess the potential of MSC administration in human ALI/ARDS. MSC administration restored lung endothelial permeability, lung water level, and alveolar fluid clearance in lungs injured with endotoxin. MSCs also decreased inflammatory infiltration [57]. A randomized control trial (SMART) is underway using MSC administration in ARDS.

## 52.9 Challenges in mesenchymal stromal cell administration in lung diseases

Despite the large body of data showing the benefits of MSCs in preclinical models, challenges remain.

### 52.9.1 Optimal dosage of mesenchymal stromal cells

The optimal dose and route of delivery of MSCs remain to be determined and may be dependent on the lung disease itself. Preclinical animal models have shown significant variation. In

rodents, a dose of $20–30 \times 10^6$ cells/kg has been used. The average dose of MSCs derived from the bone marrow was $28.3 \pm 21.8 \times 10^6$ cells/kg. In clinical trials for lung disease, $1–2 \times 10^6$ cells/kg have been used. Clinical trials on the ClinicalTrials.gov website include conditions such as IPF (NCT01385644), bronchopulmonary dysplasia (NCT01632475), and ARDS (NCT01775774).

### 52.9.2 Timing of mesenchymal stromal cell administration

In preclinical models there appears to be an optimum window period in which to use MSCs. Most studies have treated animals within 6 h of acute lung injury. The optimum time from studies utilizing different animal models suggests MSC administration within 2–3 days of lung injury. Ortiz *et al.* showed that MSCs administrated 5 days after injury did not ameliorate fibrosis [95]. Thus, MSC administration for chronic lung disease may be limited in the management of established fibrosis and tissue remodeling.

## 52.10 Summary and conclusions

Lung diseases comprise a heterogeneous group of diseases with different pathogenic mechanisms. MSCs administered intravenously enter the lung in significant numbers but only remain in the lung for a short period in time. MSCs have been shown to have a variety of potentially useful actions in diseased lungs, including reduction in inflammation and improvement in repair mechanisms.

Goals for the use of MSCs in lung disorders include:

1 Determination the disease states (if any) for which MSCs could be effective.
2 Determination of the optimal dose and frequency of MSC administration in the management of lung disorders.
3 Consideration of the use of MSCs to deliver therapeutic agents.

Taken together, many questions remain, but the journey may yield outcomes that could have a significant impact on how a number of lung diseases are managed.

## References


1 Buist AS, Vollmer WM, Sullivan SD, *et al.* The Burden of Obstructive Lung Disease Initiative (BOLD): rationale and design. *COPD* 2005;**2**(2):277–283.
2 Hubbard R. The burden of lung disease. *Thorax* 2006;**61**(7): 557–558.
3 Shaya FT, Lin PJ, Aljawadi MH, Scharf SM. Elevated economic burden in obstructive lung disease patients with concomitant sleep apnea syndrome. *Sleep Breath* 2009;**13**(4):317–323.
4 Menezes AM, Perez-Padilla R, Hallal PC, *et al.* Worldwide burden of COPD in high- and low-income countries. Part II. Burden of chronic obstructive lung disease in Latin America: the PLATINO study. *Int J Tuberc Lung Dis* 2008;**12**(7):709–712.
5 West JB. *Respiratory Physiology: The Essentials*. Lippincott Williams & Wilkins: Philadelphia, PA, 2008.
6 Moodley YP, Misso NL, Scaffidi AK, *et al.* Inverse effects of interleukin-6 on apoptosis of fibroblasts from pulmonary fibrosis and normal lungs. *Am J Respir Cell Mol Biol* 2003;**29**(4):490–498.
7 Gross TJ, Hunninghake GW. Idiopathic pulmonary fibrosis. *N Engl J Med* 2001;**345**(7):517–525.
8 Amsellem V, Gary-Bobo G, Marcos E, *et al.* Telomere dysfunction causes sustained inflammation in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 2011;**184**(12):1358–1566.
9 Dominici M, Le Blanc K, Mueller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.
10 Moodley Y, Atienza D, Manuelpillai U, *et al.* Human umbilical cord mesenchymal stem cells reduce fibrosis of bleomycin-induced lung injury. *Am J Pathol* 2009;**175**(1):303–313.
11 Moodley Y, Ilancheran S, Samuel C, *et al.* Human amnion epithelial cell transplantation abrogates lung fibrosis and augments repair. *Am J Respir Crit Care Med* 2010;**182**(5):643–651.
12 Friedenstein AJ, Chailakhjan RK, Lalykina KS. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**(4):393–403.
13 Sabatini F, Petecchia L, Tavian M, *et al.* Human bronchial fibroblasts exhibit a mesenchymal stem cell phenotype and multilineage differentiating potentialities. *Lab Invest* 2005;**85**(8):962–971.
14 Alt E, Yan Y, Gehmert S, *et al.* Fibroblasts share mesenchymal phenotypes with stem cells, but lack their differentiation and colony-forming potential. *Biol Cell* 2011;**103**(4):197–208.
15 Blasi A, Martino C, Balducci L, *et al.* Dermal fibroblasts display similar phenotypic and differentiation capacity to fat-derived mesenchymal stem cells, but differ in anti-inflammatory and angiogenic potential. *Vasc Cell* 2011;**3**(1):5.
16 Da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**(Pt 11):2204–2213.
17 Lama VN, Smith L, Badri L, *et al.* Evidence for tissue-resident mesenchymal stem cells in human adult lung from studies of transplanted allografts. *J Clin Invest* 2007;**117**(4):989–996.
18 Ricciardi M, Malpeli G, Bifari F, *et al.* Comparison of epithelial differentiation and immune regulatory properties of mesenchymal stromal cells derived from human lung and bone marrow. *PLoS ONE* 2012;**7**(5):e35639.
19 McQualter JL, Yuen K, Williams B, Bertoncello I. Evidence of an epithelial stem/progenitor cell hierarchy in the adult mouse lung. *Proc Natl Acad Sci U S A* 2010;**107**(4):1414–1419.
20 Fischer UM, Harting MT, Jimenez F, *et al.* Pulmonary passage is a major obstacle for intravenous stem cell delivery: the pulmonary first-pass effect. *Stem Cells Dev* 2009;**18**(5):683–692.
21 Schrepfer S, Deuse T, Reichenspurner H, *et al.* Stem cell transplantation: the lung barrier. *Transplant Proc* 2007;**39**(2):573–576.
22 Gholamrezanezhad A, Mirpour S, Bagheri M, *et al. In vivo* tracking of $^{111}$In-oxine labeled mesenchymal stem cells following infusion in patients with advanced cirrhosis. *Nucl Med Biol* 2011;**38**(7): 961–967.

23 Yan X, Liu Y, Han Q, *et al.* Injured microenvironment directly guides the differentiation of engrafted Flk-1$^+$ mesenchymal stem cell in lung. *Exp Hematol* 2007;**35**(9):1466–1475.

24 Gupta N, Su X, Popov B, *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *J Immunol* 2007;**179**(3):1855–1863.

25 Jacobs SA, Roobrouck VD, Verfaillie CM, Van Gool SW. Immunological characteristics of human mesenchymal stem cells and multipotent adult progenitor cells. *Immunol Cell Biol* 2013;**91**(1):32–39.

26 Huang XP, Sun Z, Miyagi Y, *et al.* Differentiation of allogeneic mesenchymal stem cells induces immunogenicity and limits their long-term benefits for myocardial repair. *Circulation* 2010;**122**(23):2419–2429.

27 Poncelet AJ, Vercruysse J, Saliez A, Gianello P. Although pig allogeneic mesenchymal stem cells are not immunogenic *in vitro,* intracardiac injection elicits an immune response *in vivo*. *Transplantation* 2007;**83**(6):783–790.

28 Beggs KJ, Lyubimov A, Borneman JN, *et al.* Immunologic consequences of multiple, high-dose administration of allogeneic mesenchymal stem cells to baboons. *Cell Transplant* 2006;**15**(8–9):711–721.

29 Nauta AJ, Westerhuis G, Kruisselbrink AB, *et al.* Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. *Blood* 2006;**108**(6):2114–2120.

30 Poggi A, Prevosto C, Zancolli M, *et al.* NKG2D and natural cytotoxicity receptors are involved in natural killer cell interaction with self-antigen presenting cells and stromal cells. *Ann N Y Acad Sci* 2007;**1109**:47–57.

31 Sotiropoulou PA, Perez SA, Gritzapis AD, *et al.* Interactions between human mesenchymal stem cells and natural killer cells. *Stem Cells* 2006;**24**(1):74–85.

32 Maggini J, Mirkin G, Bognanni I, *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE* 2010;**5**(2):e9252.

33 Raffaghello L, Bianchi G, Bertolotto M, *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells* 2008;**26**(1):151–162.

34 Brown JM, Nemeth K, Kushnir-Sukhov NM, *et al.* Bone marrow stromal cells inhibit mast cell function via a COX2-dependent mechanism. *Clin Exp Allergy* 2011;**41**(4):526–534.

35 Kim H-S, Yun J-W, Shin T-H, *et al.* Human Umbilical cord blood mesenchymal stem cell-derived $PGE_2$ and TGF-β1 alleviate atopic dermatitis by reducing mast cell degranulation. *Stem Cells* 2015;**33**(4):1254–1266.

36 Spaggiari GM, Capobianco A, Abdelrazik H, *et al.* Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 2008;**111**(3):1327–1333.

37 Zhang W, Ge W, Li C, *et al.* Effects of mesenchymal stem cells on differentiation, maturation, and function of human monocyte-derived dendritic cells. *Stem Cells Dev* 2004;**13**(3):263–271.

38 Zhang Y, Cai W, Huang Q, *et al.* Mesenchymal stem cells alleviate bacteria-induced liver injury in mice by inducing regulatory dendritic cells. *Hepatology* 2014;**59**(2):671–682.

39 Ramasamy R, Fazekasova H, Lam EW, *et al.* Mesenchymal stem cells inhibit dendritic cell differentiation and function by preventing entry into the cell cycle. *Transplantation* 2007;**83**(1):71–76.

40 Tabera S, Perez-Simon JA, Diez-Campelo M, *et al.* The effect of mesenchymal stem cells on the viability, proliferation and differentiation of B-lymphocytes. *Haematologica* 2008;**93**(9):1301–1309.

41 Corcione A, Benvenuto F, Ferretti E, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood* 2006;**107**(1):367–372.

42 Augello A, Tasso R, Negrini SM, *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol* 2005;**35**(5):1482–1490.

43 Peng Y, Chen X, Liu Q, *et al.* Mesenchymal stromal cells infusions improve refractory chronic graft versus host disease through an increase of CD5$^+$ regulatory B cells producing interleukin 10. *Leukemia* 2015;**29**(3):636–646.

44 Di Nicola M, Carlo-Stella C, Magni M, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**(10):3838–3843.

45 Ren G, Zhang L, Zhao X, *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell* 2008;**2**(2):141–150.

46 Selmani Z, Naji A, Zidi I, *et al.* Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4$^+$CD25$^{high}$FOXP3$^+$ regulatory T cells. *Stem Cells* 2008;**26**(1):212–222.

47 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.

48 Meisel R, Zibert A, Laryea M, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* 2004;**103**(12):4619–4621.

49 Luz-Crawford P, Noel D, Fernandez X, *et al.* Mesenchymal stem cells repress Th17 molecular program through the PD-1 pathway. *PLoS ONE* 2012;**7**(9):e45272.

50 Duffy MM, Pindjakova J, Hanley SA, *et al.* Mesenchymal stem cell inhibition of T-helper 17 cell- differentiation is triggered by cell-cell contact and mediated by prostaglandin E2 via the EP4 receptor. *Eur J Immunol* 2011;**41**(10):2840–2851.

51 Hof-Nahor I, Leshansky L, Shivtiel S, *et al.* Human mesenchymal stem cells shift CD8$^+$ T cells towards a suppressive phenotype by inducing tolerogenic monocytes. *J Cell Sci* 2012;**125**(Pt 19):4640–4650.

52 Spees JL, Olson SD, Ylostalo J, *et al.* Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma. *Proc Natl Acad Sci U S A* 2003;**100**(5):2397–2402.

53 Horwitz EM, Gordon PL, Koo WK, *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 2002;**99**(13):8932–8937.

54 Zhao LR, Duan WM, Reyes M, *et al.* Human bone marrow stem cells exhibit neural phenotypes and ameliorate neurological deficits after grafting into the ischemic brain of rats. *Exp Neurol* 2002;**174**(1):11–20.

55 Zhang J, Jia XH, Xu ZW, *et al.* Improved mesenchymal stem cell survival in ischemic heart through electroacupuncture. *Chin J Integr Med* 2013;**19**(8):573–581.

56 Morigi M, Imberti B, Zoja C, *et al.* Mesenchymal stem cells are renotropic, helping to repair the kidney and improve function in acute renal failure. *J Am Soc Nephrol* 2004;**15**(7):1794–1804.

57 Lee JW, Fang X, Gupta N, *et al.* Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the *ex vivo* perfused human lung. *Proc Natl Acad Sci U S A* 2009;**106**(38):16357–16362.

58 Abdel Aziz M, Wassef M, Rashed L, *et al.* Mesenchymal stem cells therapy in acute renal failure: possible role of hepatocyte growth factor. *J Stem Cell Res Ther* 2011;**1**:109. doi: 10.4172/2157-7633.1000109

59 Gyorgy B, Szabo TG, Pasztoi M, *et al.* Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci* 2011;**68**(16):2667–2688.

60 Bruno S, Grange C, Deregibus MC, *et al.* Mesenchymal stem cell-derived microvesicles protect against acute tubular injury. *J Am Soc Nephrol* 2009;**20**(5):1053–1067.

61 Zhu YG, Feng XM, Abbott J, *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells* 2014;**32**(1):116–125.

62 Gennai S, Monsel A, Hao Q, *et al.* Microvesicles derived from human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation. *Am J Transplant* 2015;**15**(9):2404–2412.

63 Islam MN, Das SR, Emin MT, *et al.* Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* 2012;**18**(5):759–765.

64 Eirin A, Riester SM, Zhu XY, *et al.* MicroRNA and mRNA cargo of extracellular vesicles from porcine adipose tissue-derived mesenchymal stem cells. *Gene* 2014;**551**(1):55–64.

65 Lee C, Mitsialis SA, Aslam M, *et al.* Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation* 2012;**126**(22):2601–2611.

66 Courboulin A, Paulin R, Giguere NJ, *et al.* Role for miR-204 in human pulmonary arterial hypertension. *J Exp Med* 2011;**208**(3):535–548.

67 Sage EK, Kolluri KK, McNulty K, *et al.* Systemic but not topical TRAIL-expressing mesenchymal stem cells reduce tumour growth in malignant mesothelioma. *Thorax* 2014;**69**(7):638–647.

68 Myers TJ, Granero-Molto F, Longobardi L, *et al.* Mesenchymal stem cells at the intersection of cell and gene therapy. *Exp Opin Biol Ther* 2010;**10**(12):1663–1679.

69 Xia L, McDaniel JM, Yago T, *et al.* Surface fucosylation of human cord blood cells augments binding to P-selectin and E-selectin and enhances engraftment in bone marrow. *Blood* 2004;**104**(10):3091–3096.

70 Sarkar D, Zhao W, Gupta A, *et al.* Cell surface engineering of mesenchymal stem cells. *Methods Mol Biol* 2011;**698**:505–523.

71 Kean TJ, Lin P, Caplan AI, Dennis JE. MSCs: delivery routes and engraftment, cell-targeting strategies, and immune modulation. *Stem Cells Int* 2013;**2013**:732742.

72 Lan YW, Choo KB, Chen CM, *et al.* Hypoxia-preconditioned mesenchymal stem cells attenuate bleomycin-induced pulmonary fibrosis. *Stem Cell Res Ther* 2015;**6**(1):97.

73 Kanki-Horimoto S, Horimoto H, Mieno S, *et al.* Implantation of mesenchymal stem cells overexpressing endothelial nitric oxide synthase improves right ventricular impairments caused by pulmonary hypertension. *Circulation* 2006;**114**(1 Suppl):I181–I185.

74 He ZX, Wang HW, Shang F, *et al.* Administrating bone marrow mesenchymal stem cells to treat the experimental pulmonary arterial hypertension in rats. *Zhonghua Yi Xue Za Zhi* 2009;**89**(30):2110–2115 (in Chinese).

75 Luan Y, Zhang ZH, Wei DE, *et al.* Implantation of mesenchymal stem cells improves right ventricular impairments caused by experimental pulmonary hypertension. *Am J Med Sci* 2012;**343**(5):402–406.

76 Jungebluth P, Luedde M, Ferrer E, *et al.* Mesenchymal stem cells restore lung function by recruiting resident and nonresident proteins. *Cell Transplant* 2011;**20**(10):1561–1574.

77 Baber SR, Deng W, Master RG, *et al.* Intratracheal mesenchymal stem cell administration attenuates monocrotaline-induced pulmonary hypertension and endothelial dysfunction. *Am J Physiol Heart Circ Physiol* 2007;**292**(2):H1120–H1128.

78 Umar S, de Visser YP, Steendijk P, *et al.* Allogenic stem cell therapy improves right ventricular function by improving lung pathology in rats with pulmonary hypertension. *Am J Physiol Heart Circ Physiol* 2009;**297**(5):H1606–H1616.

79 Takemiya K, Kai H, Yasukawa H, *et al.* Mesenchymal stem cell-based prostacyclin synthase gene therapy for pulmonary hypertension rats. *Basic Res Cardiol* 2010;**105**(3):409–417.

80 Liang OD, Mitsialis SA, Chang MS, *et al.* Mesenchymal stromal cells expressing heme oxygenase-1 reverse pulmonary hypertension. *Stem Cells* 2011;**29**(1):99–107.

81 Bonfield TL, Koloze M, Lennon DP, *et al.* Human mesenchymal stem cells suppress chronic airway inflammation in the murine ovalbumin asthma model. *Am J Physiol Lung Cell Mol Physiol* 2010;**299**(6):L760–L770.

82 Nemeth K, Keane-Myers A, Brown JM, *et al.* Bone marrow stromal cells use TGF-β to suppress allergic responses in a mouse model of ragweed-induced asthma. *Proc Natl Acad Sci U S A* 2010;**107**(12):5652–5657.

83 Firinci F, Karaman M, Baran Y, *et al.* Mesenchymal stem cells ameliorate the histopathological changes in a murine model of chronic asthma. *Int Immunopharmacol* 2011;**11**(8):1120–1126.

84 Goodwin M, Sueblinvong V, Eisenhauer P, *et al.* Bone marrow-derived mesenchymal stromal cells inhibit Th2-mediated allergic airways inflammation in mice. *Stem Cells* 2011;**29**(7):1137–1148.

85 Lee SH, Jang AS, Kwon JH, *et al.* Mesenchymal stem cell transfer suppresses airway remodeling in a toluene diisocyanate-induced murine asthma model. *Allergy Asthma Immunol Res* 2011;**3**(3):205–211.

86 Van Haaften T, Byrne R, Bonnet S, *et al.* Airway delivery of mesenchymal stem cells prevents arrested alveolar growth in neonatal lung injury in rats. *Am J Respir Crit Care Med* 2009;**180**(11):1131–1142.

87 Zhang X, Wang H, Shi Y, *et al.* Role of bone marrow-derived mesenchymal stem cells in the prevention of hyperoxia-induced lung injury in newborn mice. *Cell Biol Int* 2012;**36**(6):589–594.

88 Hansmann G, Fernandez-Gonzalez A, Aslam M, *et al.* Mesenchymal stem cell-mediated reversal of bronchopulmonary dysplasia and associated pulmonary hypertension. *Pulm Circ* 2012;**2**(2):170–181.

89 Tropea KA, Leder E, Aslam M, *et al*. Bronchioalveolar stem cells increase after mesenchymal stromal cell treatment in a mouse model of bronchopulmonary dysplasia. *Am J Physiol Lung Cell Mol Physiol* 2012;**302**(9):L829–L837.

90 Waszak P, Alphonse R, Vadivel A, *et al*. Preconditioning enhances the paracrine effect of mesenchymal stem cells in preventing oxygen-induced neonatal lung injury in rats. *Stem Cells Dev* 2012;**21**(15):2789–2797.

91 Aslam M, Baveja R, Liang OD, *et al*. Bone marrow stromal cells attenuate lung injury in a murine model of neonatal chronic lung disease. *Am J Respir Crit Care Med* 2009;**180**(11):1122–1130.

92 King TE, Jr., Schwarz MI, Brown K, *et al*. Idiopathic pulmonary fibrosis: relationship between histopathologic features and mortality. *Am J Respir Crit Care Med* 2001;**164**(6):1025–1032.

93 Raghu G, Weycker D, Edelsberg J, *et al*. Incidence and prevalence of idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med* 2006;**174**(7):810–816.

94 Hart SP. Understanding CT patterns in idiopathic pulmonary fibrosis. *Lancet Respir Med* 2014;**2**(4):249–250.

95 Ortiz LA, Gambelli F, McBride C, *et al*. Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci USA* 2003;**100**(14):8407–8411.

96 Cui A, Dai HP, Dai JW, *et al*. Effects of bone marrow mesenchymal stem cells on bleomycin induced pulmonary fibrosis in rats. *Zhonghua Jie He He Hu Xi Za Zhi* 2007;**30**(9):677–682 (in Chinese).

97 Zhao F, Zhang YF, Liu YG, *et al*. Therapeutic effects of bone marrow-derived mesenchymal stem cells engraftment on bleomycin-induced lung injury in rats. *Transplant Proc* 2008;**40**(5):1700–1705.

98 Bitencourt CS, Pereira PA, Ramos SG, *et al*. Hyaluronidase recruits mesenchymal-like cells to the lung and ameliorates fibrosis. *Fibrogenesis Tissue Repair* 2011;**4**(1):3.

99 Chambers DC, Enever D, Ilic N, *et al*. A phase 1b study of placenta-derived stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* 2014;**19**:1013–1018.

100 Halbert RJ, Natoli JL, Gano A, *et al*. Global burden of COPD: systematic review and meta-analysis. *Eur Respir J* 2006;**28**(3):523–532.

101 Kohler D, Fischer J, Raschke F, Schonhofer B. Usefulness of GOLD classification of COPD severity. *Thorax* 2003;**58**(9):825.

102 Rennard SI. Pathogenesis of COPD. *Clin Cornerstone* 2003;**5**(1):11–16.

103 Guan XJ, Song L, Han FF, *et al*. Mesenchymal stem cells protect cigarette smoke-damaged lung and pulmonary function partly via VEGF–VEGF receptors. *J Cell Biochem* 2013;**114**(2):323–335.

104 Zhen G, Liu H, Gu N, *et al*. Mesenchymal stem cells transplantation protects against rat pulmonary emphysema. *Frontiers Biosci* 2008;**13**:3415–3422.

105 Liu HM, Zhen GH, Zhang ZX, *et al*. Effects of bone marrow mesenchymal stem cells transplantation on the apoptosis of alveolar wall cells in papain and Co60-induced pulmonary emphysema rats. *Zhongguo Ying Yong Sheng Li Xue Za Zhi* 2008;**24**(2):210–214 (in Chinese).

106 Zhen G, Xue Z, Zhao J, *et al*. Mesenchymal stem cell transplantation increases expression of vascular endothelial growth factor in papain-induced emphysematous lungs and inhibits apoptosis of lung cells. *Cytotherapy* 2010;**12**(5):605–614.

107 Kirkham PA, Barnes PJ. Oxidative stress in COPD. *Chest* 2013;**144**(1):266–273.

108 Van Eeden SF, Sin DD. Oxidative stress in chronic obstructive pulmonary disease: a lung and systemic process. *Can Respir J* 2013;**20**(1):27–29.

109 Li J, Li D, Liu X, *et al*. Human umbilical cord mesenchymal stem cells reduce systemic inflammation and attenuate LPS-induced acute lung injury in rats. *J Inflamm (Lond)* 2012;**9**(1):33.

110 Jin Z, Pan X, Zhou K, *et al*. Biological effects and mechanisms of action of mesenchymal stem cell therapy in chronic obstructive pulmonary disease. *J Int Med Res* 2015;**43**(3):303–310.

111 Weiss DJ, Casaburi R, Flannery R, *et al*. A placebo-controlled, randomized trial of mesenchymal stem cells in COPD. *Chest* 2013;**143**(6):1590–1598.

112 Brower RG, Ware LB, Berthiaume Y, Matthay MA. Treatment of ARDS. *Chest* 2001;**120**(4):1347–1367.

113 Ferguson ND, Fan E, Camporota L, *et al*. The Berlin definition of ARDS: an expanded rationale, justification, and supplementary material. *Intensive Care Med* 2012;**38**(10):1573–1582.

114 Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;**342**(18):1334–1349.

115 Petty TL, Ashbaugh DG. The adult respiratory distress syndrome. Clinical features, factors influencing prognosis and principles of management. *Chest* 1971;**60**(3):233–239.

116 Leaver SK, Evans TW. Acute respiratory distress syndrome. *BMJ* 2007;**335**(7616):389–394.

117 Clark JG, Milberg JA, Steinberg KP, Hudson LD. Type III procollagen peptide in the adult respiratory distress syndrome. Association of increased peptide levels in bronchoalveolar lavage fluid with increased risk for death. *Ann Intern Med* 1995;**122**(1):17–23.

118 The Acute Respiratory Distress Syndrome Network. Ventilation with lower tidal volumes as compared with traditional tidal volumes for acute lung injury and the acute respiratory distress syndrome. *N Engl J Med* 2000;**342**(18):1301–1308.

119 National Heart, Lung, and Blood Institute Acute Respiratory Distress Syndrome (ARDS) Clinical Trials Network, Wiedemann HP, Wheeler AP, et al. Comparison of two fluid-management strategies in acute lung injury. *N Engl J Med* 2006;**354**(24):2564–2575.

120 Gupta N, Krasnodembskaya A, Kapetanaki M, *et al*. Mesenchymal stem cells enhance survival and bacterial clearance in murine *Escherichia coli* pneumonia. *Thorax* 2012;**67**(6):533–539.

121 Kim ES, Chang YS, Choi SJ, *et al*. Intratracheal transplantation of human umbilical cord blood-derived mesenchymal stem cells attenuates *Escherichia coli*-induced acute lung injury in mice. *Respir Res* 2011;**12**:108.

122 Asmussen S, Ito H, Traber DL, *et al*. Human mesenchymal stem cells reduce the severity of acute lung injury in a sheep model of bacterial pneumonia. *Thorax* 2014;**69**(9):819–825.

123 Liang ZX, Sun JP, Wang P, *et al*. Bone marrow-derived mesenchymal stem cells protect rats from endotoxin-induced acute lung injury. *Chin Med J* 2011;**124**(17):2715–2722.

124 Tai WL, Dong ZX, Zhang DD, Wang DH. Therapeutic effect of intravenous bone marrow-derived mesenchymal stem cell transplantation on early-stage LPS-induced acute lung injury in mice. *Nan Fang Yi Ke Da Xue Xue Bao* 2012;**32**(3):283–290.

125 Mei SH, McCarter SD, Deng Y, *et al*. Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Med* 2007;**4**(9):e269.

126 Xu J, Woods CR, Mora AL, *et al*. Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *Am J Physiol Lung Cell Mol Physiol* 2007;**293**(1): L131–L141.

127 Ionescu L, Byrne RN, van Haaften T, *et al*. Stem cell conditioned medium improves acute lung injury in mice: *in vivo* evidence for stem cell paracrine action. *Am J Physiol Lung Cell Mol Physiol* 2012;**303**(11):L967–L977.

128 Krasnodembskaya A, Song Y, Fang X, *et al*. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010;**28**(12): 2229–2238.

129 Sun J, Han ZB, Liao W, *et al*. Intrapulmonary delivery of human umbilical cord mesenchymal stem cells attenuates acute lung injury by expanding $CD4^{+}CD25^{+}$ Forkhead Boxp3 $(FOXP3)^{+}$ regulatory T cells and balancing anti- and pro-inflammatory factors. *Cell Physiol Biochem* 2011;**27**(5):587–596.

130 Zhu YG, Hao Q, Monsel A, *et al*. Adult stem cells for acute lung injury: remaining questions and concerns. *Respirology* 2013;**18** (5):744–756.

131 Wang Y, Folkesson HG, Jayr C, *et al*. Alveolar epithelial fluid transport can be simultaneously upregulated by both KGF and β-agonist therapy. *J Appl Physiol* 1999;**87**(5):1852–1860.

132 Guery BP, Mason CM, Dobard EP, *et al*. Keratinocyte growth factor increases transalveolar sodium reabsorption in normal and injured rat lungs. *Am J Respir Crit Care Med* 1997;**155**(5):1777–1784.

133 Planes C, Blot-Chabaud M, Matthay MA, *et al*. Hypoxia and $\beta_2$-agonists regulate cell surface expression of the epithelial sodium channel in native alveolar epithelial cells. *J Biol Chem* 2002;**277** (49):47318–47324.

134 Gamble JR, Drew J, Trezise L, *et al*. Angiopoietin-1 is an anti-permeability and anti-inflammatory agent *in vitro* and targets cell junctions. *Circ Res* 2000;**87**(7):603–607.

135 Thurston G, Suri C, Smith K, *et al*. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* 1999;**286**(5449):2511–2514.

136 Nemeth K, Mayer B, Mezey E. Modulation of bone marrow stromal cell functions in infectious diseases by toll-like receptor ligands. *J Mol Med* 2010;**88**(1):5–10.

137 Mei SH, Haitsma JJ, Dos Santos CC, *et al*. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *Am J Respir Crit Care Med* 2010; **182**(8):1047–1057.

CHAPTER 53

# The role of mesenchymal stromal cells in diseases of the lung

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## 53.1 Introduction

The lungs are the perfect organ for the intravenous (IV) administration of mesenchymal stromal cells (MSCs) since they localize there within seconds of administration (see Chapters 22 and 23). They lodge there for approximately 3 days. This is likely due to their large size. In a cell suspension *in vitro* murine MSCs have a diameter of 15–19 μm [1] and murine pulmonary capillaries have a diameter of approximately 4 μm [1]. Human MSCs in a single cell suspension have a diameter of 100–120 μm [2], while human pulmonary capillaries have a diameter of 8–30 μm [3]. A human lymphocyte by comparison has a diameter of 7–8 μm and traverses the pulmonary capillary endothelial network unimpeded. The size of MSCs, however, does not prevent their subsequent transit out of the lungs, and they then home preferentially to sites of inflammation. An example of this is shown in Figure 22.5D and E. Immediately prior to IV injection a small incision was made on the right side of the abdominal wall and this area preferentially attracted the injected MSCs after their passage through the lungs.

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MSCs secrete a number of proteins that have anti-inflammatory, antiapoptosis, and immunomodulatory functions that may mediate some of their effects. The MSC secretome is described in detail in Chapters 1, 18, and 19. The way that paracrine function is mediated by tunneling nanotubes is described in Chapter 21. The MSC exosome is described in Chapters 1, 20, and 38.

This chapter will review lung diseases in which MSCs have been shown to be beneficial in preclinical murine models and will review clinical trials using MSCs for diseases of the lung.

## 53.2 Pulmonary fibrosis

Pulmonary fibrosis can be a primary disease of unknown etiology (idiopathic pulmonary fibrosis) or it may secondary to other diseases. Most of these are classified as interstitial lung diseases. Examples include autoimmune disorders, viral infections, and bacterial infections, including tuberculosis, which may cause fibrotic changes in both lungs, especially in the upper lobes. However, pulmonary fibrosis can also appear without any known cause and is diagnosed by lung biopsy and after all primary causes

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have been excluded. Diseases and conditions that may cause pulmonary fibrosis as a secondary effect include inhalation of environmental and occupational pollutants (e.g., asbestosis, silicosis, and exposure to certain gases), hypersensitivity pneumonitis (most often resulting from inhaling dust contaminated with bacterial, fungal, or animal products), some connective tissue diseases (e.g., rheumatoid arthritis, systemic lupus erythematosus, scleroderma – also known as systemic sclerosis – and sarcoidosis), and some medications (e.g., bleomycin, busulfan, methotrexate, and amiodarone).

Pulmonary fibrosis involves a gradual replacement of normal lung parenchyma with fibrotic tissue. The replacement of normal lung with scar tissue causes an irreversible decrease in oxygen diffusion capacity, and the resulting stiffness or decreased compliance makes pulmonary fibrosis a restrictive lung disease. Pulmonary fibrosis is perpetuated by aberrant wound healing rather than chronic inflammation. Idiopathic pulmonary fibrosis occurs in middle age without a known cause. It is fatal without lung transplantation. The end result shows scarring and honeycombing of lung tissue, as shown in the example taken at autopsy in Figure 53.1.

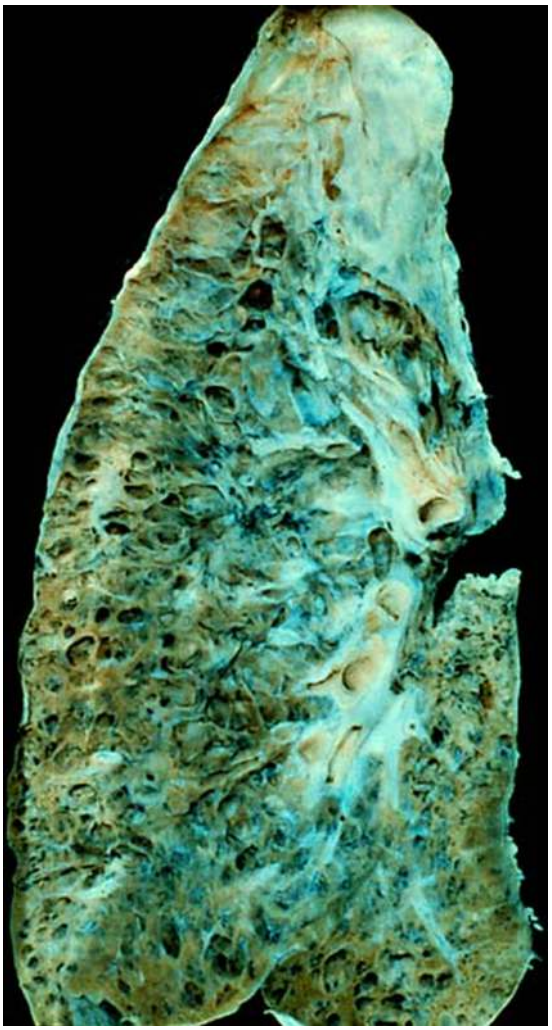


**Figure 53.1** Lung with end-stage pulmonary fibrosis at autopsy. *Source*: https://commons.wikimedia.org/wiki/File:End-stage_interstitial_lung_disease_(honeycomb_lung).jpg.

### 53.2.1 Animal models

One of the earliest models investigating MSCs in lung disease was a murine model of pulmonary fibrosis induced by the chemotherapeutic cytotoxic agent bleomycin [4]. It causes inflammatory and fibrotic reactions within a short period of time. An initial rise in the proinflammatory cytokines interleukin (IL)-1, tumor necrosis factor-α, IL-6, and interferon-γ is followed approximately 9 days later by increased expression of the profibrotic markers transforming growth factor-β1, fibronectin, and procollagen-1 with a peak around day 14 [5]. MSCs have been shown to be effective in ameliorating this disorder. Ortiz *et al.* demonstrated that bone marrow-derived murine MSCs homed to lung in response to injury, adopted an epithelium-like phenotype and reduced inflammation and collagen deposition in lung tissue of mice challenged with bleomycin [6]. In another study it was thought that MSCs produced their effect by downregulating matrix metalloproteinase 9, tissue inhibitor of metalloproteinases 1, interferon-γ, and transforming growth factor-β [7].

### 53.2.2 Clinical trials of mesenchymal stromal cells

Chambers *et al.* [8] performed a single-center, nonrandomized, dose-escalation phase 1 trial in patients with moderately severe idiopathic pulmonary fibrosis as defined by a diffusing capacity for carbon monoxide (DLCO) of ≥25% of predicted and a forced vital capacity (FVC) of ≥50% of predicted. They received intravenously either $1 \times 10^6$ ($n = 4$) or $2 \times 10^6$ ($n = 4$) MSCs/kg isolated from the term placenta of an unrelated, human-leukocyte-antigen-unmatched donor. They were followed for 6 months with lung function testing (FVC and DLCO), a 6-min walk distance, and computed tomography of the chest. Eight patients, four males and four females with an age range from 61 to 71 years and with a median (interquartile range) FVC 60% (52.5–74.5%) and DLCO 34.5% (29.5–40%) of predicted values were treated. Both dose schedules were well tolerated with only minor and transient acute adverse effects. The MSC infusion was associated with a transient (1% (0–2%)) fall in $SaO_2$ after 15 min, but without any hemodynamic changes. At 6 months, FVC, DLCO, 6-min walk distance, and computed tomography fibrosis scores were unchanged compared with baseline. There was no evidence of worsening fibrosis. The value of this study was that it demonstrated the safety of IV infusion of these doses of MSCs into people whose lung function was already significantly impaired.

## 53.3 Asthma

Asthma is a common chronic inflammatory disease of the airways characterized by variable and recurring symptoms,

reversible airflow obstruction, hyperresponsiveness of the small airways, and bronchospasm. Common symptoms include wheezing, coughing, chest tightness, and shortness of breath. Asthma is thought to be caused by a combination of genetic and environmental factors. Its diagnosis is usually based on the pattern of symptoms, response to therapy over time, and spirometry. It is clinically classified according to the frequency of symptoms, forced expiratory volume in 1 s (FEV1), and peak expiratory flow rate (Figure 53.2).

Asthma may also be classified as atopic (extrinsic) or non-atopic (intrinsic), where atopy refers to a predisposition toward developing type 1 hypersensitivity reactions. The combination of asthma, eczema, and allergic rhinitis is known as atopy. Treatment of acute symptoms is usually with an inhaled short-acting β-2 agonist (such as salbutamol), sometimes in combination with inhaled corticosteroids. In more severe cases oral corticosteroids may be required. In very severe cases, IV corticosteroids and hospitalization may be required. Symptoms can be prevented by avoiding triggers, such as allergens and irritants, and by the use of inhaled corticosteroids. Long-acting β agonists or antileukotriene agents may be used in addition to inhaled corticosteroids if asthma symptoms remain uncontrolled. Asthma is the result of chronic inflammation of the airways that subsequently results in increased contractility of the surrounding smooth muscles (Figure 53.3).

This among, other factors, leads to bouts of narrowing of the airway and the classic symptom of wheezing. The narrowing is typically reversible with or without treatment. Occasionally the airways themselves change. Typical changes in the airways include an increase in eosinophils and thickening of the lamina reticularis. Chronically, the airways' smooth muscle may increase in size along with an increase in the numbers of mucous glands. Other cell types involved include T cells, macrophages, and neutrophils. There may also be involvement of other components of the immune system, including cytokines,



**Figure 53.2** Peak flow meters are used to measure the peak expiratory flow rate, important in both monitoring and diagnosing asthma. (See color plate section.) *Source*: https://commons.wikimedia.org/wiki/File:Two_Peak_Flow_Meters.jpg#/media/File:Two_Peak_Flow_Meters.jpg.

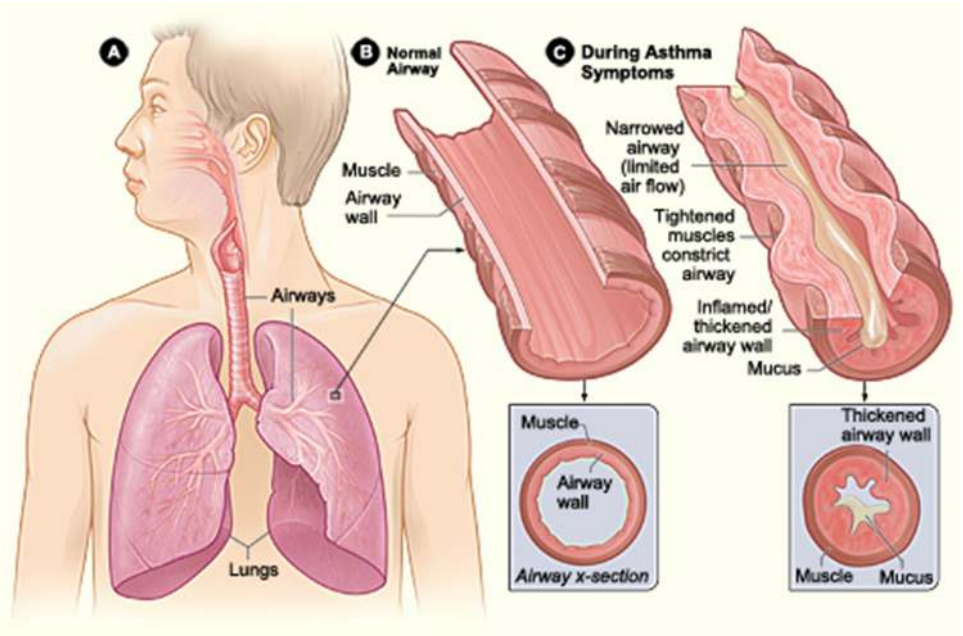



**Figure 53.3** (A) Location of the lungs and airways in the body. (B) Cross-section of a normal airway. (C) Cross-section of an airway during asthma symptoms. *Source*: NIH.

chemokines, histamine, and leukotrienes. A particularly severe form of asthma that is refractory to conventional treatment occurs in approximately 5% of people with asthma and may be associated with a marked neutrophilic infiltrate in the small airways.

A detailed description of the immunobiology of asthma is given in Chapter 39.

The commonest cause of allergic asthma in developed countries is the house dust mite (HDM). Asthma can also be precipitated by exercise, immersion in cold water, aspirin, and alcohol. Occupational asthma also occurs, and several hundred different agents have been implicated, with the most common being isocyanates, grain and wood dust, colophony, soldering flux, latex, animals, and aldehydes. Jobs associated with the highest risk of problems include those who spray paint, bakers and those who process food, nurses, chemical workers, those who work with animals, welders, hairdressers, and timber workers.

The occurrence of asthma has increased significantly since the 1970s. In 2011, 235–300 million people globally were diagnosed with asthma, and it caused 250 000 deaths.

### 53.3.1 Preclinical models

We conducted a study in which BALB/c mice were rendered allergic to HDM, the commonest allergen in Australia of allergic asthma. No adjuvant treatment was used [9]. It is known that allergic asthma is underpinned by T helper 2 (Th2) inflammation. Redundancy in Th2 cytokine function and production by both innate and adaptive immune cells suggested that strategies aimed at immunomodulation might prove beneficial. We sought to determine whether administration of MSCs to HDM-sensitized mice would suppress the development of Th2 inflammation and airway hyperresponsiveness (AHR) following HDM challenge. We found that the IV administration of allogeneic donor MSCs 1 h prior to allergen challenge significantly attenuated the features of allergic asthma in this model, including tissue eosinophilia, Th2 cytokine (IL-5 and IL-13) levels in bronchoalveolar lavage fluid, and AHR. The number of infiltrating type 2 innate lymphoid cells was not affected by MSC transfer, suggesting that MSCs may modulate the adaptive arm of Th2 immunity. The effect of MSC administration was long-lasting: all features of allergic airways disease were significantly suppressed in response to a second round of HDM challenge 4 weeks after MSC administration. We also observed that MSCs decreased the release of epithelial-cell-derived alarmins IL-1α and high-mobility group box-1 in an IL-1 receptor antagonist-dependent manner. This in turn decreased the expression of the pro-Th2 cytokine IL-25 and reduced the number of activated and antigen-acquiring $CD11c^+$ $CD11b^+$ dendritic cells in the lung and mediastinal lymph nodes. These findings suggested that MSC administration can ameliorate allergic airway inflammation by blunting the amplification of epithelial-derived inflammatory cytokines induced by exposure to HDM and may offer long-term protection against Th2-mediated allergic airway inflammation and AHR.

### 53.3.2 Clinical trials of mesenchymal stromal cells

The following trial was listed as recruiting when "mesenchymal stem cells and asthma" were searched in ClinicalTrials.gov: Safety and Feasibility Study of Intranasal Mesenchymal Trophic Factor (MTF) for Treatment of Asthma. No trials were found when the terms "mesenchymal stromal cells and asthma" were searched. In contrast, 4887 trials were listed when "asthma" alone was searched. When the terms "mesenchymal stem cells and lung diseases" were searched a total of 40 trials were listed.

## 53.4 Obliterative bronchiolitis

Obliterative bronchiolitis (OB), also known as bronchiolitis obliterans, is a rare and life-threatening form of nonreversible obstructive lung disease in which the bronchioles are compressed and narrowed by fibrosis and/or inflammation. OB has always been thought to be irreversible and progressive, but the use of inhaled cyclosporine has been shown to increase the FEV1 percentage, while patients are able to decrease their systemic corticosteroid doses.

### 53.4.1 Preclinical animal models

OB is a manifestation of chronic lung allograft rejection. A similar disease is a manifestation of chronic graft-versus-host disease occurring after allogeneic hematopoietic stem cell transplantation. It is characterized by small airway inflammation and occlusion by fibrous tissue. It is immune mediated. Al-Dessari and colleagues described a model of obliterative bronchiolitis occurring after lung transplantation using genetically defined miniature swine [10]. Group 1 ($n = 2$) received a left lung autograft; group 2 ($n = 7$) received a left lung allograft. Group 2 recipients were given cyclosporine, prednisone, and azathioprine for 3 months, and then immunosuppression was tapered and discontinued over 1 month. The animals were observed for an additional 5 months and then sacrificed. Lung grafts in both groups were monitored with serial bronchoalveolar lavages and transbronchial biopsies for 6 months. After sacrifice, lung grafts underwent histopathologic and immunohistochemical examination. No allograft had histologic evidence of acute rejection or peribronchiolar infiltrate during the first 3 months of immunosuppression. During the tapering period, airway changes characterized by severe peribronchiolar lymphocytic infiltrates were seen. Bronchoalveolar lavages of allografts showed significantly increased lymphocyte counts, with $CD8^+$ cells predominating. After the discontinuation of immunosuppression, transbronchial biopsy and autopsy specimens showed progressive fibrous inflammatory occlusion of bronchioles. Immunohistochemical staining demonstrated increased expression of major histocompatibility complex class II antigen on the bronchiolar epithelium and increased numbers of dendritic cells and $CD4^+$ lymphocytes. None of these changes were seen in the recipients of lung autografts (group 1). These findings indicated that OB is an

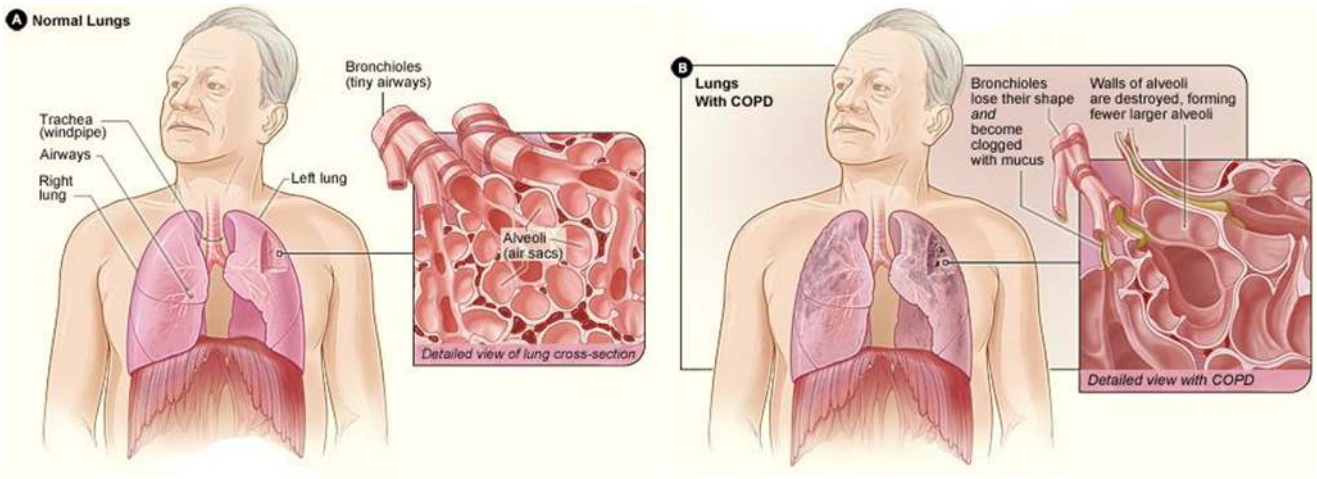



**Figure 53.4** (A) Location of the lungs and airways in the body. The inset image shows a detailed cross-section of the bronchioles and alveoli. (B) Lungs damaged by COPD. The inset image shows a detailed cross-section of the damaged bronchioles and alveolar walls. *Source*: NIH.

immunologically mediated phenomenon related to chronic graft rejection after lung transplantation. It is likely that a similar pathogenesis mediated by donor T cells present in the transplantant inoculum occurs after allogeneic hematopoietic stem cell transplantation as one manifestation of chronic graft-versus-host disease.

### 53.4.2 Clinical trials of mesenchymal stromal cells

Two trials were listed when the terms "mesenchymal stem cells and obliterative bronchiolitis" were searched in ClinicalTrials.gov. Both were in OB after lung allografting. One trial had been terminated because of loss of funding. The other was in the recruitment stage. When the terms "mesenchymal stromal cells and obliterative bronchiolitis" were searched, one additional study, again after lung transplantation, was listed as recruiting.

## 53.5 Chronic obstructive pulmonary disease and emphysema

Chronic obstructive pulmonary disease (COPD), also known as chronic obstructive airways disease, is characterized by chronically poor airflow in and out of the lungs. The main symptoms are shortness of breath, cough, and sputum production. Most people with chronic bronchitis have COPD.

Tobacco smoking is the most common cause of COPD, with other factors such as air pollution and genetics playing a smaller role. In the developing world, one of the common sources of air pollution is from poorly vented cooking and heating fires. Long-term exposure to these irritants causes an inflammatory response in the lungs, resulting in narrowing of the small airways and breakdown of lung tissue known as emphysema (Figure 53.4).

The diagnosis is based on poor airflow as measured by lung function tests. In contrast to asthma, the airflow reduction does not improve significantly with the administration of medications.

COPD can be prevented by reducing exposure to the known causes. This includes efforts to decrease rates of smoking and to improve indoor and outdoor air quality. COPD treatments include quitting smoking, influenza and pneumococcal vaccinations, rehabilitation, and often inhaled bronchodilators and corticosteroids. Some people may benefit from long-term oxygen therapy or lung transplantation.

Worldwide, COPD affects 329 million people, or nearly 5% of the population. In 2013, it resulted in 2.9 million deaths, up from 2.4 million deaths in 1990. The number of deaths is projected to increase due to higher smoking rates and an aging population in many countries. An image taken at autopsy of a human lung severely damaged by COPD is shown in Figure 53.5.




**Figure 53.5** Gross pathology of a human lung showing centrilobular-type emphysema characteristic of smoking. This close-up of the fixed, cut lung surface shows multiple cavities lined by heavy black carbon deposits. (See color plate section.) *Source*: www.cdc.gov.

### 53.5.1 Preclinical animal models

Many of the hallmark features of human COPD can be mimicked in animal models of the disease, including chronic lung inflammation with accumulation of macrophages, neutrophils, and lymphocytes, impaired lung function, emphysema, mucus hypersecretion, small airway wall thickening and remodeling with an increase in matrix components, inflammatory cells and goblet cell metaplasia in the airway wall, resulting in luminal narrowing, distortion, and obstruction by mucus, vascular remodeling, lymphoid aggregates, and pulmonary hypertension.

Churg *et al.* described an acute murine model of exposure to smoke from four cigarettes over 1 h. They observed that the resulting inflammation was associated with activation of the transcription factor nuclear factor κB and proposed that tumor necrosis factor-α, processed by matrix metalloproteinase 12, was largely responsible for the ensuing responses, especially upregulation of the vascular adhesion molecule E-selectin [11].

### 53.5.2 Clinical trials with mesenchymal stromal cells

A search of ClinicalTrials.gov using the terms "mesenchymal stem cells and chronic obstructive airways disease" showed one complete trial carried out by Mesoblast PTL, but no results were available. Using the terms "mesenchymal stem cells and emphysema," three are listed. The status of one was unknown, one was recruiting, and one was completed in 2012 but no results were available.

## 53.6 Bronchopulmonary dysplasia

Bronchopulmonary dysplasia is a chronic lung disorder of infants and children first described in 1967. It is more common in infants with low birth weight and those who receive prolonged mechanical ventilation and prolonged high oxygen delivery to treat respiratory distress syndrome. It results in significant morbidity and mortality. Management includes surfactant, antenatal glucocorticoid therapy, and less aggressive mechanical ventilation. Prolonged high oxygen delivery in premature infants causes necrotizing bronchiolitis and alveolar septal injury, with inflammation and scarring. This results in hypoxemia. Current practice using surfactant and high-frequency ventilation and oxygen supplementation results in a much milder injury without necrotizing bronchiolitis or alveolar septal fibrosis. Instead, there are usually uniformly dilated acini with thin alveolar septa and little or no interstitial fibrosis.

### 53.6.1 Preclinical animal models

Newborn animal models employing both premature and term animals have been used over the years to study various components of bronchopulmonary dysplasia. The similarities to lung injury in human premature infants include an acute inflammatory response with the production of cytokines, chemokines, and growth factors that have been implicated in human disease, abnormal pulmonary function, disordered lung architecture, alveolar simplification, development of fibrosis, and abnormal vascular growth factor expression [12].

### 53.6.2 Clinical trials using mesenchymal stromal cells

When the terms "mesenchymal stem cells and bronchopulmonary dysplasia" were searched in ClinicalTrials.gov, eight trials were listed. Their status was as follows: one was not yet recruiting, one was of unknown status, three were recruiting, two were active but not yet recruiting, and one was completed. No results were given for the completed trial. Six of the eight trials used PNEUMOSTEM®, a preparation of allogeneic umbilical-cord-blood-derived MSCs.

## 53.7 Acute respiratory distress syndrome and acute lung injury

Acute respiratory distress syndrome (ARDS), acute lung injury, adult respiratory distress syndrome, or shock lung is a severe, life-threatening medical condition characterized by widespread inflammation in the lungs. While ARDS may be triggered by trauma or lung infection, it is usually the result of sepsis (bacteria in the bloodstream).

ARDS is a disease of the microscopic air sacs of the lungs (alveoli) that leads to a decreased exchange of oxygen and carbon dioxide. ARDS is associated with several pathologic changes: the release of proinflammatory proteins, breakdown of the cells lining the blood vessels in the lungs, loss of surfactant, leading to increased surface tension in the lung, fluid accumulation in the lung, and excessive fibrous connective tissue formation.

The syndrome has a high mortality of between 20 and 50%.

The signs and symptoms of ARDS usually begins within 72 h of the initial insult or injury to the lung and include shortness of breath, tachypnea (fast breathing rate), and a low oxygen level in the blood. Chest X-ray frequently demonstrates generalized infiltrates or opacities in both lungs, which represent fluid accumulation in the lungs.

The predisposing factors of ARDS are numerous and varied. Common causes include sepsis, pneumonia, trauma, multiple blood transfusions, babesiosis, lung contusion, aspiration of stomach contents, and drug abuse or overdose. (Babesiosis is a malaria-like parasitic disease caused by infection with *Babesia*, a genus of protozoal piroplasms.) Other causes of ARDS include burns, pancreatitis, near drowning, or the inhalation of chemical irritants such as smoke, phosgene, or chlorine gas.

ARDS is an acute injury to the lungs that results in the flooding of the alveoli, the small air sacs within the lungs where the exchange of oxygen and carbon dioxide occurs, partial collapse of the lungs (atelectasis), and low levels of oxygen in the blood (hypoxemia). The triggering insult to the tissue usually results in an initial release of chemical signals and other inflammatory mediators secreted by local epithelial and endothelial cells. Neutrophils and T cells quickly migrate into the inflamed

lung tissue and contribute in the amplification of the phenomenon. Typical histolopathological changes include diffuse alveolar damage and hyaline membrane formation in alveolar walls. ARDS is usually treated with mechanical ventilation in a hospital intensive care unit. Ventilation is usually delivered through orotracheal intubation, or by tracheostomy whenever prolonged ventilation (≥2 weeks) is deemed inevitable.

### 53.7.1 Preclinical animal models

There are a number of animal models of ARDS, each focusing on different aspects of the disorder: lipopolysaccharide (LPS) delivered intra-tracheally is a good model for neutrophil recruitment; acid aspiration is a good model for epithelial barrier disruption; hyperoxia is a good model for hemorrhagic injury; surfactant depletion is a good model for lung injury induced by other agents, especially mechanical ventilation.

In one of the most dramatic reports LPS (*Escherichia coli* endotoxin) was instilled into the airway of mice. After this, murine bone-marrow-derived MSCs (BM-MSCs) were instilled into the airway. Live optical studies revealed that MSCs formed connexin-43-containing gap junctional channels (GJCs) with the alveolar epithelium, releasing mitochondria-containing microvesicles that the epithelium engulfed. The presence of MSC mitochondria in the epithelium was evident optically. The mitochondrial transfer increased alveolar adenosine triphosphate. This LPS-induced acute lung injury, indicated by alveolar leukocytosis and protein leak, inhibition of surfactant secretion and high mortality, was markedly abrogated by wild type murine BM-MSCs, but not by mutant, GJC-incompetent murine BM-MSCs or by murine BM-MSCs with dysfunctional mitochondria. It demonstrated that BM-MSCs can protect against acute lung injury by reconstituting alveolar bioenergetics through connexin-43-dependent alveolar attachment and mitochondrial transfer [13].

### 53.7.2 Clinical trials with mesenchymal stromal cells

Searching Clinical Trials.gov with the terms "mesenchymal stem cells and acute respiratory distress syndrome/acute lung injury" revealed five trials. Four were recruiting and the status of the fifth was unknown. Of those recruiting, two were using adult BM-MSCs and one was using umbilical-cord-derived MSCs.

## 53.8 Conclusions

The use of mesenchymal stromal cells to treat lung disease is in its infancy. It is encouraging that MSCs administered intravenously to people with impaired lung function and pulmonary fibrosis appeared safe and did not further impair lung function by their initial (presumed) lodgment in the lungs. The results of the ongoing trials are awaited.

## References


1 Shrepfer S, Deuse T, Reichenspurner H, *et al.* 2007.Stem cell transplantation: the lung barrier. *Transplant Proc* **39**(2):573–576.
2 Barlow S, Brooke G, Chatterjee K, *et al.* 2008.Comparison of human placenta- and bone marrow-derived multipotent mesenchymal stromal cells. *Stem Cells Dev* **17**:1095–1107.
3 Freitas Jr, RA. 1999. 8.2.1.2. Arteriovenous microcirculation. In: *Nanomedicine, Volume 1: Basic Capabilities*. Landes Bioscience: Georgetown, TX.
4 Adamson IY, Bowden DH. 1974.The pathogenesis of bleomycin-induced pulmonary fibrosis in mice. *Am J Pathol* **77**(2):185–197.
5 Moeller A, Ask K, Warburton D, *et al.* 2008.The bleomycin animal model: a useful tool to investigate treatment options for idiopathic pulmonary fibrosis? *Int J Biochem Cell Biol* 2008;**40**(3):362–382.
6 Ortiz LA, Gambelli F, McBride C, *et al.* 2003.Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* **100**(14):8407–8411.
7 Yu SH, Liu LJ, Lv B, *et al.* 2015.Inhibition of bleomycin-induced pulmonary fibrosis by bone marrow-derived mesenchymal stem cells might be mediated by decreasing MMP9, TIMP-1, *INF-γ and TGF-β. Cell Biochem Funct* **33**(6):356–366.
8 Chambers DC, Enever D, Ilic N, *et al.* 2014.A phase 1b study of placenta-derived mesenchymal stromal cells in patients with idiopathic pulmonary fibrosis. *Respirology* **19**(7):1013–1018.
9 Duong KM, Eacharach J, Ullah MA, *et al.* 2015.Immunomodulation of airway epithelium cell activation by mesenchymal stromal cells ameliorates HDM-induced airway inflammation in mice. *Am J Respir Cell Mol Biol* **53**(5):615–624.
10 Al-Dossari GA, Kshettry VR, Jessurun J, Bolman III, RM. 1994. Experimental large-animal model of obliterative bronchiolitis after lung transplantation. *Ann Thorac Surg* **8**(1):34–39, discussion 39–40.
11 Churg A, Wang RD, Tai H, *et al.* 2003.Macrophage metalloelastase mediates acute cigarette smoke-induced inflammation via tumor necrosis factor-α release. *Am J Respir Crit Care Med* **167**:1083–1089.
12 D'Angio CT, Ryan RM. 2014.Animal models of bronchopulmonary dysplasia. The preterm and term rabbit models. *Am J Physiol Lung Cell Mol Physiol* **307**(12):L959–969.
13 Islam MN, Das SR, Emin MT, *et al.* 2012.Mitochondrial transfer from bone marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nat Med* **18**(5):759–765.

CHAPTER 54

# Mesenchymal stromal cells for the treatment of autoimmune diseases

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## 54.1 Cell biology of endogenous mesenchymal stromal cells

In vertebrates, the majority of postnatal hematopoiesis occurs in the marrow of the long bones. Each day the marrow of adult humans releases hundreds of millions of new blood cells into the circulation [1], but the profound proliferative capacity of this system must be kept in check by a variety of mechanisms: dysregulated marrow is the genesis of autoimmune disease and hematologic dyscrasias, both benign and malignant. The hematopoietic stem cell (HSC) is the multipotent self-renewing progenitor that gives rise to all cells of the lymphoid (T cell, B cell, natural killer cell) and myeloid (erythrocyte, monocyte, granulocyte, platelet) lineages. The HSC functions in the context of a niche populated by nonhematopoietic cells of stromal origin. These stromal elements play a central role in marrow homeostasis and are the object of our current discussion.

A bone marrow aspirate reveals the stromal tissue of mammalian bone marrow to be a complex structure, punctuated by blood cell progenitors of varying maturity and interspersed with adipocytes as well as bone-remodeling osteoclasts and osteoblasts. In the highly vascularized endosteal niche the stromal cell network surrounds and coordinates the development of HSCs. If a healthy human bone marrow aspirate is put into tissue culture plates, after a week of culture a population of plastic-adherent spindle-shaped cells will become manifest. This process of *ex vivo*

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expansion is necessary in order to generate appreciable amounts of cells, for in the native marrow true mesenchymal stem cells are vanishingly rare and can be found at a frequency of approximately 1 out of 10,000 cells [2]. By ex vivo expansion large numbers of mesenchymal stromal cells can be obtained, a progenitor population that can differentiate into a number of mesoderm-derived tissues such as adipose tissue, cartilage, and osteocytes. It is this mesenchymal progenitor feature that serves loosely as rationale for the term "stem cell"; but to prevent confusion with HSCs, and in reference to their stromal derivation, we and others refer to these as mesenchymal stromal cells, though the abbreviation "MSC" is used for both mesenchymal stem cells and mesenchymal stromal cells. In this chapter "MSC" will used solely to denote mesenchymal *stromal* cells.

### 54.1.1 Mesenchymal stromal cells coordinate hematopoietic stem cell development

Within the vascularized marrow compartment, fate mapping and parabiosis experiments have elucidated different MSC classes that interact with HSCs to coordinate hematopoiesis [3–7]. Although MSC surface and intracellular markers vary in the literature, such variance is thought to reflect the different microenvironments in which the cells reside (e.g., the skin, lung, fat, marrow) rather than distinct cell lineages [7]. One of the more well-described marrow-resident MSCs is the C–X–C motif chemokine ligand 12 (CXCL12)-abundant reticular (CAR) stromal cell. Named for their reticuloid processes as well as their chemokine production, CAR stromal cells have been shown to be essential for functional hematopoiesis. (Note that these CAR stromal cells are totally unrelated to T cells that have been genetically engineered to express specific receptors on their surface called chimeric antigen receptors and which are also known as CAR cells).

CXCL12 is produced by stromal cells in the marrow and is also required for normal lymphopoiesis – the development of a functionally mature T and B cell compartment. The use of conditional knockout mice has elucidated the connection between CAR stromal cells and committed lymphoid progenitors in the marrow. Using a *Cre–lox* system Greenbaum *et al.* showed that, when CXCL12 is deleted only from CAR stromal cells, animals exhibit a sharp drop in the numbers of pre-pro B cells and have fewer marrow committed lymphoid progenitors [6]. CAR stromal cells are also principal sources of stem cell factor (SCF, also known as kit ligand), a cytokine that is presented on the stromal cell membrane. SCF is the ligand for the receptor tyrosine kinase c-Kit (CD117), which transduces important pro-survival signals to developing HSCs [8,9]. As such, CAR stromal-cell-derived signals are indispensable for normal hematopoiesis. Using an inducible knockout system in mice, Omatsu *et al.* have shown that short-term ablation of CAR stromal cells decreases the self-renewing cell cycling of HSCs, as well as reducing the numbers of both lymphoid and erythroid progenitor cells [4].

Other work has described a rare population of CXCL12-producing cells referred to as nestin$^+$ stromal cells. These cells are named for their co-expression of the intermediate filament protein nestin and are found in perivascular bundles in close apposition to both HSCs and catecholaminergic nerve fibers. The mobilization of HSC progeny from the marrow has been observed to oscillate with circadian rhythms delivered via the sympathetic nervous system [3,10]. Work by Méndez-Ferrer has shown that these nestin$^+$ stromal cells express the $\beta_3$-adrenergic receptor and that, upon selective depletion, the mobilization and development of HSCs is significantly impaired [5]. These findings illustrate the ability of MSCs to coordinate hematopoiesis by integrating paracrine signals with long-range autonomic control (see also Chapter 32).

### 54.1.2 Mesenchymal stromal cells and central tolerance in the bone marrow

Lymphopoeisis is a complex process wherein T and B cell progenitors generate a unique antigen receptor via the recombination of hundreds of different gene segments. Immunologists have calculated that on the order of $10^{18}$ different antigen receptors could be made stochastically, although certain mechanisms (allelic exclusion, the preponderance of certain T cell receptor β-chains) revise this number downward [11]. Nonetheless, gene segment recombination endows a healthy vertebrate with an extremely diverse immune repertoire, poised to respond to a vast array of proteins, lipids, or carbohydrates. As a result of this combinatorial diversity, occasional lymphocytes are generated whose receptors target self-antigens. Left unchecked, such T or B cells may escape to the periphery, and under certain conditions initiate a pathologic autoimmune process. The body has a number of mechanisms whereby it evades autoimmune disease, but these can be grouped into two general classes: central tolerance and peripheral tolerance. By central tolerance, we refer to events occurring in the primary lymphoid organs – the bone marrow, thymus or spleen – whereas peripheral tolerance may be induced anywhere in the body that leukocytes are found.

In the marrow, lymphoid progenitors with newly generated antigen receptors are in constant contact with the stromal cell network. It is important to note that although lymphoid progenitors begin this process in the marrow, T cells migrate to the thymus in order to undergo maturation. In a process termed positive selection, new lymphocytes ensure that their receptor is capable of recognizing peptides presented by endogenous antigen-presenting cells (APCs).

In negative selection, lymphocytes that vigorously autoreact to self-antigens are neutralized to so-called clonal ignorance, anergy, or undergo apoptosis, here termed deletion. In the thymus, signals from both local tissue-resident and marrow-derived cells guide the developing T cells through the positive and negative selection processes [12]. Medullary thymic epithelial cells (mTECs) express the transcription factor autoimmune regulator (AIRE), enabling the cells to express self-derived antigens. Research has shown that mTECs can also transfer this antigen for indirect presentation by thymus-resident dendritic cells [13]. In the thymus, autoreactive T cells can be

converted to so-called natural regulatory T cells (Tregs), be deleted, or otherwise deprived of survival signals and become anergic. Figure 54.1 shows the structure of the AIRE molecule.

Studies using human cells as well as murine models have shown that MSCs can also be isolated from the thymus, and that they contribute to the development of a normal T cell compartment [14,15]. Toward the coordination of lymphopoiesis, MSCs, like professional APCs such as dendritic cells and macrophages, can express the diverse human leukocyte antigen (HLA) molecules: class I, presenting mainly intracellular-derived peptides, and class II, presenting extracellular-derived peptides. If the lymphocyte is unable to recognize, or if it autoreacts too vigorously to the HLA-presented peptides, that lymphocyte clone is deprived of pro-survival signals (e.g., CXCL12 and other growth factors) and is deleted.

These mechanisms of central tolerance, enforced by the thymus, suffice to keep a healthy individual free of autoimmune disease. Autoreactive T cells can be tolerized to self-antigen in the periphery, thereby becoming "induced" Tregs. The precise mechanisms by which these conversions occur, where they occur, and the principal inducers of Treg conversion are areas of intense interest, with changes in cellular metabolism and co-stimulatory and co-inhibitory surface molecules all being investigated [16,17].

In addition to classes I and II, less variable HLA molecules are expressed by MSCs, including HLA-G. Initially discovered on trophoblastic tissues in the placenta, HLA-G prevents maternal immune infiltration of the hemi-allogeneic fetus. When expressed by MSCs, HLA-G confers an immunosuppressive signal by activating immunotyrosine inhibitory motifs on lymphocytes and monocytes. In the marrow, HLA-G signaling dampens the activity of bone-remodeling osteoclasts that are derived from monocytes, whereas in the periphery lymphocytes respond to HLA-G on membranous vesicles [18,19].

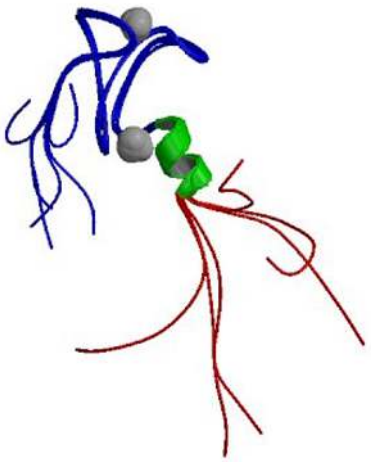


**Figure 54.1** Protein data base rendering based on 1xwh of the structure of the transcription factor AIRE. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:PBB_Protein_AIRE_image.jpg#/media/File:PBB_Protein_AIRE_image.jpg.

*Ex vivo* MSCs have been defined using conventional flow cytometric techniques by the International Society for Cellular Therapy (ISCT) [20]. These cells must stain positively for CD73 (ecto-5′-nucleotidase), CD90, and CD105 (endoglin), but lack staining for the following lymphomyeloid markers: CD11b, CD14, CD19, CD34, CD45, CD79a, or of major histocompatibility complex (MHC) class II [21]. One other surface marker that has garnered considerable attention regarding MSC functionality is the surface marker CD271. Also known as low-affinity nerve growth factor receptor, expression of CD271 has been observed on primary MSCs that also produce large amounts of CXCL12 (the aforementioned CAR cells). $CD271^+$ MSCs have been shown to support the development of HSCs *in vitro* [22]; additionally, mouse models of HSC–MSC co-transplantation demonstrate CD271 as a prospective marker for the engraftment-promoting qualities of MSCs [23]. Apart from these markers, when studied in their endogenous setting MSCs can present other differences in surface markers, perhaps reflective of the varied cytokine milieu in peripheral tissues or the marrow. It must be stated that MSCs can be isolated from a variety of nonhematopoietic tissues, including lung, fat, kidney, skin, and muscle. Indeed, some reports indicate that MSCs can be derived and propagated from any organ tissue, and that the amount of MSCs present is proportional to the blood vessel density per end-organ [24]. The role of MSCs as precursors for angiogenesis has become appreciated as recent reports show a shared phenotype with vascular pericytes. Though beyond the scope of present discussion of immune-mediated application, MSCs have been much explored in the field of regenerative medicine, particularly in cardiovascular and wound healing models [25–29].

## 54.2 Cell biology of mesenchymal stromal cells in culture

Since their initial discovery in bone marrow, MSCs have been studied by translational scientists in attempts to harness their regenerative and immunomodulatory abilities. The progenitor nature of MSCs, and their proven ability to differentiate into cartilage, bone, and fat cells, has spurred research efforts to regenerate these tissues. A number of studies have utilized MSCs to regrow musculoskeletal tissues, including craniofacial defects [30], repair of ruptured tendons [31], or to correct the congenital bone disease osteogenesis imperfecta [32]. The regenerative nature of MSCs has not been without controversy, however, as published reports have claimed the ability to repair transected spinal cords (see Chapter 49), myocardial infarcts (see Chapter 45), and even autism. Such controversy may speak to the variability inherent to performing *in vitro* differentiation with progenitor cells, in which different laboratory culture media and cell-processing protocols can be at variance.

### 54.2.1 Mesenchymal stromal cells and B cell immunosuppression

Whereas the regenerative capacity of MSCs may remain a question of tissue culture recipes, the immunomodulatory capabilities of these cells have been borne out in a variety of assay systems. From a teleological perspective, the role of MSCs in hematopoiesis affords us the best lens to view the mechanisms by which stromal cells suppress the proliferation and effector function of potentially pathologic leukocytes. After an immature B cell has undergone gene recombination and expresses its novel antigen receptor, marrow stromal cells mediate positive and negative selection. Through this process autoreactive B cells are either silenced or deleted, thereby maintaining central tolerance. For autoreactive B cells that do escape the marrow, mechanisms of peripheral tolerance exist to limit immunopathology. Given the complexities of observing central and peripheral tolerance *in situ*, a number of techniques have been developed to model how MSCs and pathologic B cells interact.

Our group explored a mouse model of hemophilia A, a coagulopathy that arises from an inherited deficiency in clotting factor VIII (FVIII) [33]. After years of FVIII replacement therapy, it is common for human patients to develop allo-antibodies to FVIII, complicating treatment and causing significant morbidity. Both *in vivo* and *in vitro* techniques demonstrated that murine MSCs induced plasma cells to re-express paired box 5 via the protein-phosphatase-dependent inhibition of signal transducer and activator of transcription (STAT)3, causing a systemic decrease in anti-FVIII antibodies. Our protocol was repeated in healthy mice immunized to ovalbumin protein, after which we observed similarly decreased anti-ovalbumin-protein immunoglobulin (Ig) production by B cells. Other studies using B cells and MSCs from healthy human donors have shown that MSC co-culture attenuates the production of IgA, IgM, and IgG via *in vitro* assay systems [34]. A study by Ma *et al.* explored the utility of MSC administration for idiopathic thrombocytopenic purpura (ITP), a hematologic disease characterized by spontaneous bleeding, petechiae, and purpura [35]. Although not all cases of ITP are proven autoimmune in nature, both T and B cells with specificity for platelet surface antigens have been implicated in its pathogenesis [36]. Using *ex vivo* culture systems of primary cells from ITP patients, Ma *et al.* show a marked decrement in platelet destruction after MSC co-culture, although they were unable to pinpoint a specific causative relationship, instead citing a number of possibilities.

### 54.2.2 Mesenchymal stromal cell and T cell co-culture assays

Different *in vitro* systems have been utilized to observe the mechanisms whereby MSCs can regulate, suppress or limit the inflammatory damage of pathologic T cells. One of the principal ways that MSC-mediated immunosuppression can be observed is by co-culture with peripheral blood mononuclear cells (PBMCs) or purified T cells. In order to activate the T cells toward proliferation and effector differentiation, the T cell markers CD3 and CD28 must be ligand-stimulated. These stimuli reproduce the so-called Signal 1 and Signal 2 of T cell activation: Signal 1, in which a T cell receptor encounters its cognate antigen presented by the HLA molecule of an APC, and Signal 2, whereby CD80 or CD86 on the APC delivers co-stimulation to the CD28 on the T cell. Whether affixed to beads or in solution, anti-CD3/anti-CD28 stimulation of PBMCs or purified T cells induces activation and proliferation that is polyclonal in nature. We and others have used this technique in combination with *in vitro* co-culture to demonstrate the suppressive effects mediated by MSCs [37–39]. Other techniques for polyclonal activation of T cells include the use of mitogenic stimuli, such as phytohemagglutinin [40] and the superantigen staphylococcal enterotoxin B [39].

To assess proliferation, and thereby judge the degree of MSC immunosuppression, different groups have employed the generational dilution of carboxyfluorescein succinimidyl ester [41], an increase in $^{3}$H-thymidine incorporation [41], or intracellular flow cytometric staining for Ki67 [39]. The classical mixed lymphocyte reaction (MLR) has also been utilized to assess the degree by which MSCs modulate T cell allo-proliferation. Given a high degree of HLA mismatch, responder T cells proliferate in an allo-specific fashion, generationally diluting their carboxyfluorescein succinimidyl ester concentration. MSCs can be plated beneath the MLR, allowing the researcher to tease apart tolerance-conferring mechanisms [42].

MSCs have been shown to suppress the proliferation of activated T cells using a variety of mechanisms, and both cell-to-cell contact and soluble factors have been implicated. Soluble factors include prostaglandin $E_2$, transforming growth factor-β (TGF-β), hepatocyte growth factor, and HLA-G, released on membranous bodies as described earlier. These and other mechanisms have been reviewed by others [20,21,43–45], so our discussion will instead focus on translationally important findings whereby stromal cells suppress the activity of T cells.

For researchers in transplantation and autoimmune disease, the word "tolerance" evokes the categorical good; immunosuppressive pharmacotherapies are thus employed to avert rejection of allografted organs or host tissues undergoing autoimmune cellular infiltration. However, tolerance can also be maladaptive, a consequence engendered by the stromal environment in which benign tumors become malignant cancers. By understanding the mechanisms of cancer-associated maladaptive tolerance, immunologists stand to gain valuable insight for next-generation immunomodulatory therapies.

## 54.3 Immunosuppression: lessons from oncology

It is now appreciated that cancer is not one syndrome, but rather a collection of diseases marked by dysregulated proliferation of the body's endogenous cells. The last century has advanced our understanding of carcinogenic toxins, inherited or acquired

mutations, and chronic inflammatory changes that can all incite cells to become cancerous. In the process, one or all of the following systems becomes dysregulated: a cell can undergo loss of function (e.g., of a tumor-suppressing factor like the retinoblastoma gene *RB*), or it can gain a competetive growth advantage (e.g., the constitutive growth triggered by *BCR-ABL*, the oncogene responsible for chronic myeloid leukemia), or it can become ignorant to apoptotic signals (e.g., mutations in the B cell lymphoma gene *BCL-2*), or lastly it gains the ability to metastasize beyond its normal niche (e.g., by the loss of E-cadherin) [46]. These changes may not occur sequentially in every cancer, but *in vitro* each is sufficient for a human cell to acquire immortality. In the healthy body it is appreciated that such dysregulations occur at a basal level, but are kept in check by a variety of homeostatic mechanisms. The concept of immune surveillance has been described as the mechanisms by which circulating lymphocytes detect premalignant changes and subsequently kill the mutated cell [47]. A number of studies have demonstrated the ability of lymphocytes to perform tumoricidal immune surveillance [48,49]. However, in instances where cancer does arise, recent discoveries indicate that tumor stromal tissue exerts immunosuppressive mechanisms that neuter the potentially cancer-fighting effects of tumor-infiltrating lymphocytes (TILs) [50].

### 54.3.1 Programmed death ligand 1 and immunosuppression by tumors

After receiving Signal 1 and Signal 2 and being exposed to inflammatory cytokines, newly-activated T cells ($CD4^+$ T helper cells and $CD8^+$ cytotoxic lymphocytes) upregulate adhesion molecules and then traffic to inflamed areas by following chemokine gradients. The activation process for T cells involves the maturation of effector function (i.e., the ability to release cytokines such as interferon (IFN)-γ or granzyme B), after which they lose dependence on the co-stimulatory Signal 2. For example, when cytotoxic lymphocytes arrive at an inflamed area, they serially kill any cell that displays the cognate antigen–HLA complex. In addition to gaining effector function, T cell activation induces the upregulation of inhibitory receptors that include programmed death-1 (PD-1) [51]. Although we have described the process of T cell activation, it is important to note that PD-1 expression is also upregulated on activated B cells. PD-1 is a transmembrane protein that contains an immunotyrosine inhibitory motif, which upon ligation recruits tyrosine phosphatases that inactivate a number of signaling molecules, including spleen tyrosine kinase, zeta-chain-associated protein kinase 70, and phosphatidylinositide 3-kinase [51]. Such changes induce the anergy or apoptosis of T cells and B cells, tempering the late immune response toward resolution. The structure of programmed death ligand 1 (PD-L1) is shown in Figure 54.2.

The principal ligands for PD-1 are PD-L1 (also called B7 homolog and CD274) and PD-L2 (also called B7 dendritic cell and CD273). Early studies of PD-L1 indicated that the molecule was commonly found on primary human cancer cells [52], although subsequent work has also located the molecule on benign hematopoeitic and nonhematopoietic cells [51]. Likewise, PD-L2, first identified on human cancer cell lines [53], has been since identified on APCs and some nonhematopoietic cells [51]. The finding that PD-L1 was associated with cancerous human tissues generated a flurry of research in the first decade of the new millennium, as scientists explored new avenues of cancer immunotherapy. Antibodies to blockade PD-1 and PD-L1 have shown great promise in preclinical models [54,55], later spurring the development of clinical trials for cancer immunotherapy [56–58]. In late 2014, the US Food and Drug Administration approved pembrolizumab, a monoclonal antibody drug targeting PD-1, as the newest therapeutic agent for metastatic melanoma [59].

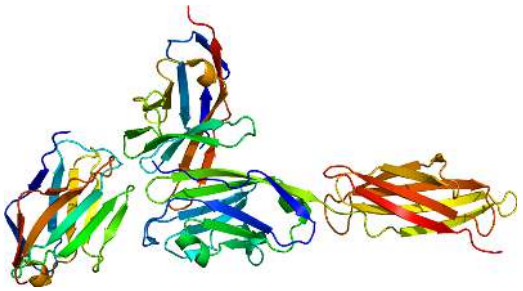


**Figure 54.2** Structure of PD-L1 (CD274). The rendering is based on PyMOL rendering of the Protein Data Bank 3BIK. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Protein_CD274_PDB_3BIK.png#/media/File:Protein_CD274_PDB_3BIK.png.

### 54.3.2 Programmed death ligand 1 and immunosuppression by mesenchymal stromal cells

Among the contact-dependent factors implicated in immunosuppression by MSCs, PD-L1 has emerged as a potentially important mediator. As discussed earlier, the role of PD-L1 has been explored in oncology research and found to dampen the effects of tumoricidal lymphocytes. Such findings have furthered efforts to improve immunomodulatory MSC therapy, via a deepened mechanistic understanding of immune tolerance. In 2005, just a few years after the first characterizations of PD-1, surface expression of PD-L1 by marrow-derived MSCs was described to mediate immunosuppression [60]. Augello *et al.* showed that resting, marrow-derived MSCs only contained mRNA for PD-L1, but could be induced to express the protein on the cell surface by inflammatory stimuli. These researchers showed that MSCs inhibited the proliferation of both B cells and T cells in an MLR or after phytohemagglutinin stimulation, and that this effect could be reversed by a blocking antibody to PD-L1. A 2008 study used MLR, mitogens, and also anti-CD3/anti-CD28 stimulation to show that lymphocyte-derived IFN-γ was responsible for the induction of PD-L1 on the surface of MSCs [61].

Other studies have further elucidated the role of PD-L1 in MSC immunosuppression. In a 2012 study, Luz-Crawford *et al.*

explored how MSCs utilize PD-L1 to inhibit both proliferation and effector capacity of T cells [62]. Using established protocols for *in vitro* T cell skewing, they generated inflammatory T helper (Th)1 and Th17 cells, confirmed by canonical transcription factors and cytokine expression (T-bet and *RORγt*, and IFN-γ and interleukin (IL)-17 respectively). They showed that MSCs are able to suppress cytokine release from both types of inflammatory T cells, which was reversed by antibody blockade of PD-L1. Of particular note was the finding that although soluble factors play a role in the immunosuppression, Th17 cells were especially sensitive to contact-dependent inhibition by PD-L1. Subsequent work by the same researchers suggested that MSCs can reprogram Th17 cells into Tregs [63], though the exact role of PD-L1 remains to be fully described. The crystallographic structure of retinoic acid receptor-related orphan receptor gamma (RORγ) is shown in Figure 54.3.

In a 2014 report our research group explored the differential effects of MSC immunosuppression after inhibition of indoleamine 2,3-dioxygenase (IDO) or PD-L1 [39]. Primary human MSCs were shown to be capable of inhibiting both the proliferation and effector function (cytokine release) of T cells after treatment with the superantigen staphylococcal enterotoxin B or anti-CD3/anti-CD28 stimulation. We showed that although IDO was associated with suppression, treatment with the enzymatic inhibitor 1-methyl-tryptophan did not fully reverse this effect. However, small-interfering-RNA-mediated knockdown of PD-L1 or PD-L2 was shown to reverse the inhibitory potential of MSCs. These results suggest that MSCs may rely on a number of factors – soluble factors, IDO catalysis, and PD-L1/PD-1 signaling – to mediate their immunosuppression by complementary nonoverlapping mechanisms.

### 54.3.3 Indoleamine 2,3-dioxygenase and immunosuppression by tumors

IDO is an important enzyme that is known to be associated with the conferrence of immunologic tolerance. It catalyzes the rate-limiting step in the biochemical degradation of tryptophan, yielding a group of metabolites collectively termed kynurenines [64]. A 2012 report identified IDO in trophoblast-derived macrophages, where it is associated with the maintenance of maternal–fetal tolerance [65]. IDO-expressing dendritic cells have likewise been found to be tolerance-inducing in a heart transplantation model [66]. Although these recent discoveries speak to the tolerogenic stimuli sought by the transplant field, the enzymatic activity of IDO was first indirectly observed in the field of oncology. In a 1956 study, patients with advanced bladder cancer were found to have abnormally high excretion of kynurenines, which could be modulated by supplementation of tryptophan or other chemical analogs [67]. Viewed through a modern lens, it is likely that IDO-expressing cells in the cancerous bladder wall augmented local tryptophan degradation, rendering any TILs impotent in their attempt to eradicate the tumor.

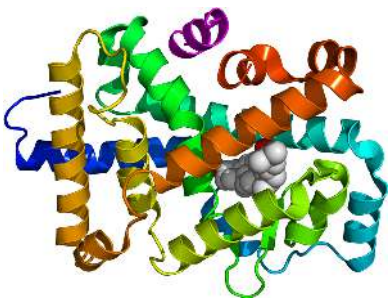


**Figure 54.3** Crystallographic structure of the ligand binding domain of RORγ (rainbow colored: N-terminus, blue; C-terminus, red) complexed with 25-hydroxycholesterol (space-filling model (carbon, white; oxygen, red) and the nuclear receptor coactivator 2 (magneta). (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:RORC_3L0L.png#/media/File:RORC_3L0L.png.

Various models have been proposed to explain how IDO can suppress the immune response and block the antitumor effect of TILs. One model holds that local deprivation of tryptophan prevents the phosphorylation of T cell receptor zeta chains, thereby preventing further activation of T cells [43]. Other studies have examined the role of IDO-generated kynurenines and how they may act directly or indirectly to reprogram effector T cells into tolerogenic Tregs [68]. Perhaps as an echo of the 1956 cancer studies, enzymatic inhibitors of IDO have been developed as an effort to abrogate its downstream immunosuppressive effects. The most promising of these inhibitors is 1-methyl-tryptophan, a structural analog that blocks the catalytic site of IDO, and is being tested in clinical trials for breast cancer, brain tumors and melanoma (http://www.clinicaltrials.gov). As such studies continue, the mechanistic switches that govern maladaptive tolerance to cancer will better inform future IDO-targeted therapies.

### 54.3.4 Indoleamine 2,3-dioxygenase and immunosuppression by mesenchymal stromal cells

Although not basally expressed in resting MSCs, upon treatment with IFN-γ the expression of IDO increases greatly in MSCs, as confirmed by mRNA levels and Western blot protein analysis [69,70] (see Section 54.4 on cytokine licensing of MSCs). A number of studies have shown that in human MSCs, the IDO enzyme is a key correlate of immunosuppressive ability [71,72]. Using bone-marrow-derived MSCs from a variety of different donors, we showed that immunosuppressive potency was correlated with the expression of IDO protein [72]. Others have explored the downstream effects of IDO-catalyzed changes in target cells and noted decreased levels of tryptophan as well as increased production of kynurenines [37]. The mechanisms whereby MSCs and kynurenines together suppress leukocyte inflammation are an area of active research [45]. In a 2011 study our group showed a link between IDO catalysis and MSC reprogramming of inflammatory macrophages (conventional or M1 macrophages) into anti-inflammatory (M2) macrophages. The contributions of M2 macrophages have become increasingly

appreciated as key players in wound healing and postinflammatory resolution [45]. Such reprogramming of peripheral macrophages may be a recapitulation of MSCs in the marrow, which are known to modulate the bone-resorptive activity of monocyte-derived osteoclasts [18]. Crosstalk between MSCs and M2 macrophages has been demonstrated by a number of studies [73–75], further suggesting this axis of immunosuppression may be important for *in vivo* efficacy.

In addition to monocytes, IDO catalysis has been found to an important factor in the reprogramming of inflammatory T cells into a regulatory phenotype. This was first suggested in a 2004 report in which MSCs were shown to inhibit the proliferation of T cells in a human MLR by IDO-catalyzed production of kynurenines [76]. These studies have been expanded through the use of animal models in which organs are co-transplanted with allogeneic MSCs [77]. The presence of IDO and the increased production of kynurenines were both shown to be associated with long-term graft tolerance as well as increased frequency of both peripheral and tissue-resident Tregs [78].

## 54.4 Mesenchymal stromal cell response to inflammatory signals: licensing and integration

Having considered the cellular biology of MSCs observed via *in vitro* methodologies, as well as appreciating their endogenous role in the normal hematopoietic niche, we now turn to how MSCs' functionality is altered by inflammatory signals. Much of the basic and clinical research occurring with MSCs explores these cells' unique immunomodulation, and how it changes before, during, and after *in vitro* culture expansion. Tissue culture experiments are vital to the furtherance of all MSC biology work, as rigorous reductionist methods teach us with each new data point obtained. When such work is predicated on endogenous observations, it affords a better perspective on how MSCs function in patients currently being treated with these cells.

We described earlier the role of MSCs in the hematopoietic niche, where they integrate a variety of signaling paradigms – CXCL12, SCF, and adrenergic stimuli – to regulate the development of lymphoid and myeloid cells that defend and oxygenate all tissues. In addition, MSCs have been observed to exert immune control in the peripheral tissues, suppressing immune activation and tipping the inflammatory milieu back toward therapeutically beneficial resolution. As stromal cells, MSCs are poised to respond to environmental cues in the marrow, adipose tissue, or other vascularized tissues, and thence exert their immunomodulatory abilities [7]. The mechanisms by which MSCs sense inflammation, integrate stimuli, and modulate the immune response depend upon basic cellular biology. That is to say, the response of MSCs is determined by the identity of activating ligands, the presence of the relevant receptor, and the activation state of diverse adaptors that transduce, amplify, or otherwise allow MSCs to coordinate the local immune response.

As tissue-resident stromal cells, MSCs can respond to inflammatory stimuli through a diversity of receptors. One of the most important classes of innate sensing molecules possessed by MSCs are the highly conserved pattern recognition receptors (PRRs). These are surface or intracellular sensing molecules that detect inflammatory changes early in the course of infection or injury. MSCs possess a number of PRRs that enable them to sense and migrate towards an inflammatory nidus and help coordinate the influx of innate and adaptive immune cells. We will sequentially discuss the effects of inflammatory signaling on MSC phenotype and behavior as it pertains to a number of different ligand classes. However, it is essential that it is understood that *in vivo* all such signaling happens simultaneously, and it is via the integration of local and long-range signals that stromal cells and leukocytes together cooperate to inflame, defend and then repair the tissue microenvironment.

Let us consider as an example a chronically nonhealing wound, such as a stasis ulcer that can arise in people with poorly controlled diabetes mellitus. Although a variety of endocrine and cardiovascular factors contribute to such pathology, over time endothelial cells and capillary-associated cells locally release inflammatory cytokines and chemokines in response to poor perfusion, leading to pathologic degradation of the extracellular matrix. One key cytokine in this process is TNF-α, which we will discuss at length later, but for now it should be noted that its local action in capillary beds causes vasodilatation, enabling the local diapedesis of leukocytes [79]. The exposed endothelium causes platelet activation, release of additional inflammatory mediators, and the formation of clots in the microvasculature, attempting containment of the insult. An increasing body of literature has appreciated the diverse secretome of platelet activation, including cytokine growth factors such as platelet-derived growth factor, vascular endothelial growth factor, and TGF-β, eicosanoids like thromboxane A2, and fibrinolytic and anti-fibrinolytic enzymes. Eicosanoids are signaling molecules made by oxidation of 20-carbon fatty acids. They exert complex control over many bodily systems, mainly in growth during and after physical activity, and in inflammation or immunity after the intake of toxic compounds or pathogens. Thromboxane A2 is a type of thromboxane that is produced by activated platelets and has prothrombotic properties. It stimulates activation of new platelets as well as increasing platelet aggregation. The structure of thromboxane A2 is shown in Figure 54.4.

### 54.4.1 Mesenchymal stromal cells and complement

The complement system is an early innate immune defense mechanism comprised of plasma proteins that interact with membrane surfaces. Elements of the complement system were first identified by their "complementary" role in antibody-mediated lysis of pathogens. However, a modern evolutionary understanding of these proteins reveals their early innate action, long before the adaptive immune system begins antibody production. Indeed, the alternative and lectin pathways demonstrate that

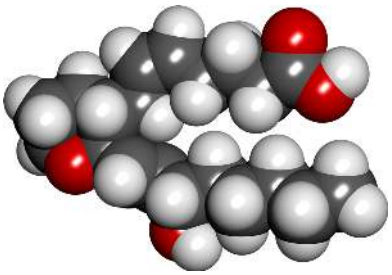


**Figure 54.4** A space-filling model of thromboxane A2. *Source:* http://commons.wikimedia.org/wiki/File:ThromboxaneA2_spacefill.png#/media/File:ThromboxaneA2_spacefill.png.

from sea urchins to humans the complement system functions to detect foreign invaders [80]. In the case of an inflammatory ulcer, a breach of the tissue with the outside environment introduces a host of pathogens. Complement proteins are deployed in the process of opsonization, to coat invading pathogens, either lysing or delivering them up to phagocytic cells for destruction. Through the deposition of complement proteins on to pathogen surfaces, the cleavage of reactive thioester bonds generates split products, such as C3a, C4a, and C5a. Listed in order of increasing inflammatory potential, these anaphylatoxins (complement peptides) ligate G-protein-coupled receptors on leukocytes to enhance phagocytosis, antigen-processing, and presentation.

MSCs have been shown to express the C3a and C5a receptors and to migrate chemotactically to *in vitro* gradients of C3a and C5a. The mechanisms of C3a receptor ligation were shown to be linked to receptor translocation and prolonged extracellular signal-regulated kinase 1/2 phosphorylation within primary human MSCs [81]. Others have shown that anaphylatoxin generation and even complement deposition on MSC surfaces is important in activating and enabling MSC immunosuppression in MLRs [82]. Moll *et al.* showed that this effect was associated with expression of the surface membrane protein CD59, which prevents complement-mediated lysis of MSCs. Together, such results suggest that MSCs are poised to respond to local complement activation and modulate the early inflammatory response to injury or infection.

### 54.4.2 Mesenchymal stromal cells and toll-like receptors

With the loss of epithelial integrity (such as in the lesions of inflammatory bowel disease – see Chapter 50), microbes entering into the wound may be of commensal origin, living in host tissues without causing overt disease, but upon entry to the subcutaneous niche become pathogenic. Such microbes are referred to as pathobionts, in that they have the ability to cause disease when in conjunction with other damage-associated markers [83]. The highly conserved molecular patterns of microbes are called pathogen-associated molecular patterns and those released from damaged tissues (such as heat shock proteins or uric acid) are called damage-associated molecular pattern molecules. A variety of immune competent cells, including MSCs, express receptors for such molecules, sensing microenvironmental changes to help coordinate the immune response [84]. In addition to the soluble defense mechanisms, including complement, ficolins (oligomeric lectins that activate the lectin pathway of complement activation), and C-reactive protein, invading pathogens are sensed by PRRs. Evolutionarily ancient in origin, PRRs recognize conserved patterns common in microbes, such as the formylated methionine residues found in bacteria, and detected by the formyl-methionyl-leucyl phenylalanine receptor.

In considering the role of PRRs in MSC biology, the best characterized class is the toll-like receptor (TLR) family. Initially discovered as innate sensing molecules in *Drosophila*, TLRs can be found on the cell surface or on intracellular membrane surfaces of a variety of vertebrate and invertebrate cells. There have been 11 TLRs described in humans and 13 in mice. These molecules are able to detect and transduce danger signals associated with pathogenic lipoproteins, lipopolysaccharide (LPS, also known as endotoxin), flagellin, double- or single-stranded RNA, and unmethylated cytosine–phosphate–guanosine DNA motifs [84,85]. It is important to note that TLRs do not just sense invading microbes but also host-derived molecules indicative of inflammatory or necrotic processes. Accordingly, TLRs have been described in the response to uric acid, heat shock proteins, intracellular cell debris, or fragments of extracellular matrix [84].

The pattern-sensing TLR domain contains about 20 leucine-rich repeats, which facilitate its ligand detection via homo- or heterodimerization. Depending on the class of ligand being sensed, a TLR will recruit different adaptor molecules to its signaling region; for example, the 200-residue toll–IL-1 receptor homology (TIR) domain, in which the toll domain is shared with the IL-1 receptor, demonstrates the importance of this cytokine in tuning and responding to inflammatory events. TLR dimerization patterns, as well as a diversity of adaptor molecules (myeloid differentiation primary response protein 88, TIR domain adaptor protein, TNF-receptor-associated factor (TRAF), and TIR-domain-containing adapter-inducing IFN-β-related adapter molecule), help to explain how only a dozen or so molecules can transduce a diversity of signals that vary according to the level of threat posed by each pathogen- or host-derived ligand. These signals feed into the nuclear factor κB (NF-κB), mitogen-activated protein kinase, or caspase pathways, resulting in immune activation, proliferation, or apoptosis [85].

The presence and function of TLRs on MSCs has been interrogated in a number of studies exploring primary murine and human MSCs derived from a variety of tissue sources. Such reports have identified some degree of expression for TLR families 1–6, although TLR3 and TLR4 are the only classes that reach expression levels comparable to that seen in hematopoietic-derived cells [86]. In one such study researchers identified that stimulation of MSCs with the well-characterized TLR4 ligand LPS abrogated the ability of MSCs to exert immunosuppressive modulation on *in vitro* T cell proliferation assays. Likewise, treatment of MSCs with poly(I:C) (a synthetic double-stranded RNA analog and TLR3 ligand) prevented the

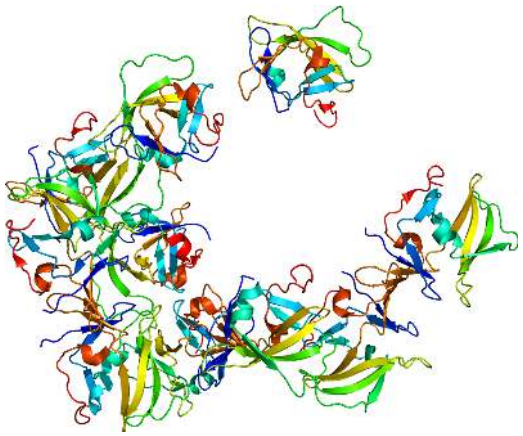


**Figure 54.5** Rendering of the structure of the DEAD box polypeptide 58 based on Protein Data Bank 2QFB. (See color plate section) *Source:* http://commons.wikimedia.org/wiki/File:Protein_DDX58_PDB_2QFB.png#/media/File:Protein_DDX58_PDB_2QFB.png.

immunosuppression observed in controls. Treatment of MSCs with either LPS or poly(I:C) induced NF-κB activation, as well MSC downregulation of the protein Jagged-1. Jagged-1 is a ligand for the T cell receptor Notch, and MSC signaling via Notch and Jagged-1 has been implicated in MSC immunosuppression of T cells [86]. Other studies show that TLR3 or TLR4 ligands cause MSCs to produce greater amounts of IL-1β, IL-6, CXCL8, chemokine (C–C motif) ligand (CCL)5, and IL-12p75 [87]. Furthermore, it was shown that treatment of MSCs with IFN-α increased expression of TLR3. Type I IFNs (IFN-α or IFN-β) function as early innate mediators and activate defenses, including protein kinase R and retinoic acid-inducible gene 1, key systems that block viral proliferation in host cells. Retinoic acid-inducible gene 1 contains an RNA helicase-DEAD (Asp–Glu–Ala–Asp) box motif and a caspase recruitment domain also known as a caspase activation and recruitment domain. The structure of DEAD box polypeptide DDX58 is shown in Figure 54.5, and a cartoon of the caspase recruitment domain RIP-associated protein with a death domain (RAIDD) is shown in Figure 54.6.

In the same experimental system we found that treatment with IFN-γ, the type II IFN, increased MSC expression of both TLR3 and TLR2. The observations that TLR ligands prevent MSC immunosuppression are nonetheless consistent with their coordinating role in the early immune response. By analogy, TLR ligands have been shown to activate dendritic cells, priming them to perform antigen-presentation to engage the adaptive immune system. Much as dendritic cells modulate the immune response, tissue-resident MSCs can be seen as responsive to microenvironmental cues, engendering early inflammation but later tipping the balance back to resolution and repair.

### 54.4.3 Interferon-γ in the immune response

The role of IFN-γ is most commonly understood as a cytokine involved in the coordination of the immune response, specifically that driven by Th1 cells. Its crystallographic structure is shown in Figure 54.7.

The first descriptions of Th1 and Th2 cells, performed by Mossman and Coffman in 1986, showed that T cell subsets respond to stimulation differently and can be characterized by their specific cytokine secretion [88]. Today, some degree of plasticity is appreciated to exist, wherein T cells may secrete multiple cytokines or be reprogrammed to different subsets. However, the role of IFN-γ derived from Th1 cells spurred many studies exploring this cytokine's effect on the immune response. Like the type I IFNs (IFN-α and IFN-β), IFN-γ was noted early on for its ability to "interfere" with viral replication. It is now thought that the Th1 response is most well adapted to a variety of intracellular pathogens, such as *Mycobacteria* and *Listeria* as well as protozoans and viruses. Since it is derived chiefly from activated T cells, it does not arise until later in the inflammatory process, after innate immune cells, both resident and immigrant, have sounded the alarm. This is an important point, for the persistence of IFN-γ in the cytokine milieu signifies ongoing inflammatory changes in the microenvironment.

In a successful immune response the immune infiltrates will defeat the pathogen, and a shift toward immune suppression, wound healing, and repair must occur, otherwise there is a risk of

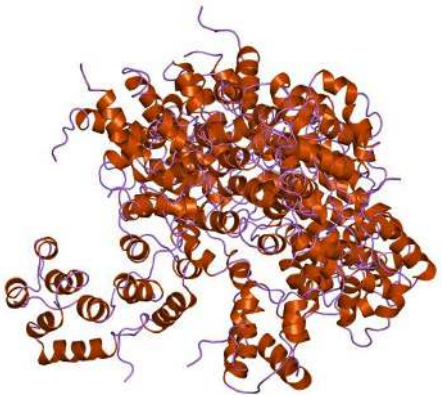


**Figure 54.6** A cartoon representing the structure of the caspase recruitment domain RAIDD. *Source:* http://commons.wikimedia.org/wiki/File:PDB_3crd_EBI.jpg#/media/File:PDB_3crd_EBI.jpg.

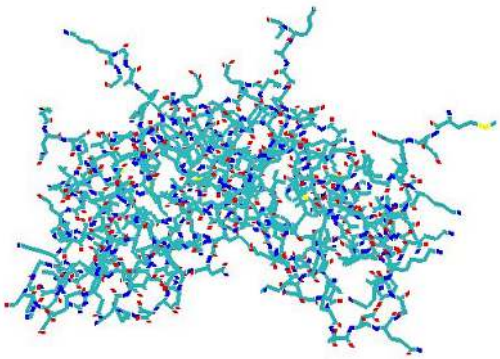


**Figure 54.7** Line representation of the crystallographic structure of IFN-γ. *Source:* http://commons.wikimedia.org/wiki/File:IFN.png#/media/File:IFN.png.

the development of a chronic nonhealing wound. From this perspective we can appreciate how and why inflammatory signals, such as IFN-γ, can deploy the anti-inflammatory effect of MSCs, both *in vitro* and *in vivo*.

### 54.4.4 Mesenchymal stromal cells and interferon-γ

In the past decade a number of studies have sought to identify the most important factors involved in immunomodulation by MSCs. Both IDO and PD-L1, described earlier, have been found to be essential in conferring on human MSCs the ability to suppress T cell proliferation and effector function. Resting MSCs – that is, cells derived from primary tissue sources and maintained in *in vitro* culture – are only partially effective at suppressing T cell proliferation. Pretreatment with an inflammatory mediator will "license," or greatly augment, the immune veto effects of the MSCs. Among the mediators described thus far, IFN-γ has been the most well explored and characterized MSC licensing agent. A number of studies have demonstrated that IFN-γ markedly enhances the ability of human MSCs to suppress allo-proliferation in MLRs, as well as mitogen-driven immune proliferation [70,89,90]. After ligating the receptor for IFN-γ (IFN-γR), Janus-family kinases 1 and 2 both associate with the intracytoplasmic tails of the receptor. Following this, STAT1 homodimerizes and is phosphorylated, enabling it to traffic to the nucleus to initiate transcription of IFN-γ-responsive genes. In MSCs, IFN-γ treatment results in STAT1 phosphorylation and the upregulation of a number of genes, including IDO. It has been shown that, in response to IFN-γ, primary human MSCs increase expression of class I HLA and begin to express *de novo* class II HLA as well as PD-L1 [39,91]. The importance of IFN-γ and IDO in enabling MSC immunosuppression has been demonstrated through the use of blocking antibodies to the IFN-γR, as well as small-interfering-RNA-mediated knockdown experiments targeting IDO, after which the MSCs are not able to suppress as well. The increased expression of HLA molecules is consistent with antigen presentation, which may be a way to recapitulate the endogenous role of MSCs as enforcers of self-tolerance as described earlier.

### 54.4.5 Tumor necrosis factor-α in the immune response

TNF-α is a cytokine that is classically associated with both acute and chronic inflammatory responses. As a component of the acute phase response, TNF-α signaling triggers containment of infection while also initiating vasodilatation in the area of inflammation. However, systemic release of TNF-α (either induced experimentally or observed in patients) results in a clinical syndrome not unlike septic shock. (Septic shock is a result of severe infection with sepsis; the latter term denotes the presence of pathogenic microorganisms in the bloodstream. Shock refers to the low blood pressure characteristic of this often catastrophic illness. It can cause multiple organ dysfunction syndrome and death.)

The discovery of TNF-α followed the characterization of a hormone known as cachectin, which had been found at high levels in the blood in patients with cachexia, a clinical syndrome with extreme muscle and fat wasting, common in late-stage cancer patients. In 1985, Bruce Beutler performed the first studies showing that TNF-α and cachectin were in fact the same molecule [92]. It is now appreciated that signaling via TNF receptors (TNFRs) is crucial for a vast array of processes, beyond the shaping of acute and chronic inflammation. As an example, TNFR signaling is essential during the development of secondary lymphoid organs, particularly those of the gut, including Peyer's patches and mesenteric lymph nodes, via the extrinsic and intrinsic transduction of apoptotic or survival cues [93,94].

We will only describe the extrinsic or death receptor pathway, as it is so vital to immune system function. Additionally, its membrane-bound activation affords a perspective on how cell-to-cell TNF signaling can induce target cell death. Upon ligation the TNFR homotrimerizes and its intracellular death domain moieties, in the case of TNFR-1, associate with TNFR-1-associated death domain (TRADD) protein. Depending on the ligand–receptor class, TRADD can then associate with Fas-associated protein with death domain and activate the initiator caspase CASP8, leading to eventual apoptosis driven by the executioner caspases CASP3, CASP6, and CASP7. Alternatively, recruitment of adaptor TRAF will instead lead to activation of the transcription factors NF-κB, as well as the kinase c-JUN, part of the activator protein 1 complex, which leads to cell survival, proliferation, or immune activation [95].

The relevance of TNFR signaling for MSC biology lies in the point that, depending on cellular context and ligand–receptor pairing, such signaling paradigms can lead to (a) activation of immune cell functions and (b) the deletion of lymphocytes during the resolution of inflammation. In order to understand the effects of inflammatory licensing of MSCs it is helpful to employ reductionist *in vitro* experiments informed by a thorough understanding of the cytokine milieu that characterizes the microenvironment. Although recognized as a key mediator of the inflammatory response, direct observations of the effects of TNF-α on MSC licensing and subsequent immunosuppression are still developing. In a 2014 paper, Dorronsoro *et al.* set out to characterize the role of TNF-α derived from T cells and its effect on the suppressive capacity of primary human MSCs [96]. They first noted that T cells activated by anti-CD3 and anti-CD28 monoclonal antibodies secreted large amounts of TNF-α in culture media and this induced robust NF-κB signaling in MSCs. Having observed both effects to correlate with immune suppression, they transduced MSCs with short hairpin RNA for the inhibitor of kappa kinase β. Forced expression of inhibitor of kappa kinase β causes constitutive repression of MSC NF-κB signaling and resultant failure to immunosuppress. In carefully designed experiments the team used transwells, anti-TNF-α antibodies, recombinant TNF-α, and a mutated form of the TNFR to show that T-cell-derived TNF-α is required for MSC immunosuppression.

Other recent work by Dorronsoro and team has explored the role of A20 zinc finger protein, which is an anti-inflammatory protein involved in signal integration downstream of TNF-α and NF-κB [97]. The protein A20 has a dual function, with a de-ubiquitinating motif as well as possessing E3 ligase activity. In cellular biology, ubiquitination is a complex process involving the enzymatic addition of ubiquitin chains to proteins, marking them for recycling via the proteosome or lysosomal degradation. (The proteasome is a multisubunit enzyme complex that plays a central role in the regulation of proteins that control cell cycle progression and apoptosis.) Multiple and redundant levels of regulation exist to tag, target, and process the ubiquitination of cell signaling proteins, any of which can go awry and lead to neurodegenerative diseases or some forms of cancer [98,99]. In APCs such as dendritic cells or macrophages, silencing of A20 leads to a hyperstimulatory effect by which these cells are able to overcome tumor-derived Treg cells, tipping the balance toward eradication by cytotoxic T cells [100]. Such studies have described A20 as an attenuator of antigen presentation, tempering the immune stimulatory effect via NF-κB degradation. Germane to MSCs, the team led by Dorronsoro found that, upon stimulation by TNF-α, MSCs rapidly degrade intracellular A20, perhaps consistent with the early inflammation of an immune response [97]. The precise regulation of TNF-α and A20 in MSC activation remains to be fully defined, but signs point toward a delicate balance of immune activation coupled to a compensatory immunosuppressive phenotype.

One last aspect of TNF-α signaling in MSCs that deserves special mention is TNF-α-stimulated gene 6 protein (TSG-6). This protein was first discovered by stimulating skin fibroblasts with TNF-α [101], but more recently its anti-inflammatory effects have been studied via its secretion from MSCs. A number of preclinical research models employing MSCs have sought to inject human MSCs into mice in order to study their engraftment and immunomodulatory potential. After the intravenous injection of MSCs in mice, the cells get lodged in the lung vasculature. However, therapeutic improvement is still observed, even after the cells are no longer present. Paracrine release of anti-inflammatory factors has been suggested to explain this effect, with TSG-6 as one likely candidate.

It was in one such study exploring the utility of MSCs to direct regeneration of myocardial tissue after infarction that Lee *et al.* [102] noted that human MSCs localized in the lungs and then upregulated mRNA for TSG-6 80-fold higher than controls. They noted that the amount of TSG-6 protein released by MSCs far exceeded that which has ever been observed from skin fibroblasts. Further experiments revealed that MSC-derived TSG-6 is involved in inhibition of matrix metalloproteinases and that its presence tips the balance toward resolution of post-infarction inflammation. One other recent study has explored the role of TSG-6 from MSCs and how this paracrine factor can temper the immune response. In a 2011 study, Choi *et al.* used a TLR2 agonist, zymosan, to induce inflammatory peritonitis in mice and then injected human MSCs intraperitoneally [103]. They noted that MSC-derived TSG-6 attenuated the inflammatory response by inhibiting NF-κB activation in peritoneal macrophages. From TSG-6, A20, and the growing number of studies on TNF-α, it is clear that this inflammatory cytokine primes MSCs, enabling them to perform their immunomodulatory function in a variety of settings.

### 54.4.6 Synergy of interferon-γ and tumor necrosis factor-α in mesenchymal stromal cells

MSC licensing has been explored through a number of studies utilizing both human and mouse MSCs in a variety of assay systems. The T cell suppression assay, described earlier, is perhaps the most reductionist method to study the pretreatments by which MSC immunomodulation can be altered. It was through such studies that Ren *et al.* demonstrated a synergistic effect after treating murine MSCs with both IFN-γ and TNF-α [104]. In this 2008 study, murine MSCs were found to depend on IFN-γ signals for activation, as MSCs lacking the IFN-γ receptor were unable to suppress T cell proliferation. It is important to note in the MSC field that murine MSCs do not utilize IDO at all. Rather, the enzyme best correlated with IFN-γ-induced licensing is inducible nitric oxide synthase. Ren *et al.* demonstrated that upon treatment with IFN-γ, plus addition of either TNF-α or IL-1, murine MSCs induced the highest levels of inducible nitric oxide synthase and chemoattracted and suppressed the proliferation of T cells more robustly than with pretreatment by a single proinflammatory cytokine. In 2010 our research group sought to explore the connection between synergistic cytokine licensing and the antigen-presenting capacity of MSCs [105]. As mentioned earlier, MSCs may be induced to express both class I and class II HLA molecules and have also been observed to perform cross-presentation, akin to dendritic cells. On treating MSCs with both IFN-γ and TNF-α we observed enhanced antigen-presenting capacity as well as reduced activation and proliferation among co-cultured T cells. As others have noted, and we described, Ren and team showed the source of these cytokines to be the activated T cells, leading to a feedback loop in which the inflammatory milieu primes MSCs into a regulatory, immunosuppressive phenotype. A schema summarizing our group's interpretation of MSC licensing is shown in Figure 54.8.

## 54.5 Strength of signal and integration

Some controversy has arisen regarding MSCs in clinical trials, as results have not been uniform in replicating promising *in vitro* findings. We and others have pointed out that this may be due to differences in cell source and processing between studies [106]. Such challenges could be inherent to cell therapeutics, suggesting they will remain the purview of academic or biomedical research centers equipped for such protocols.

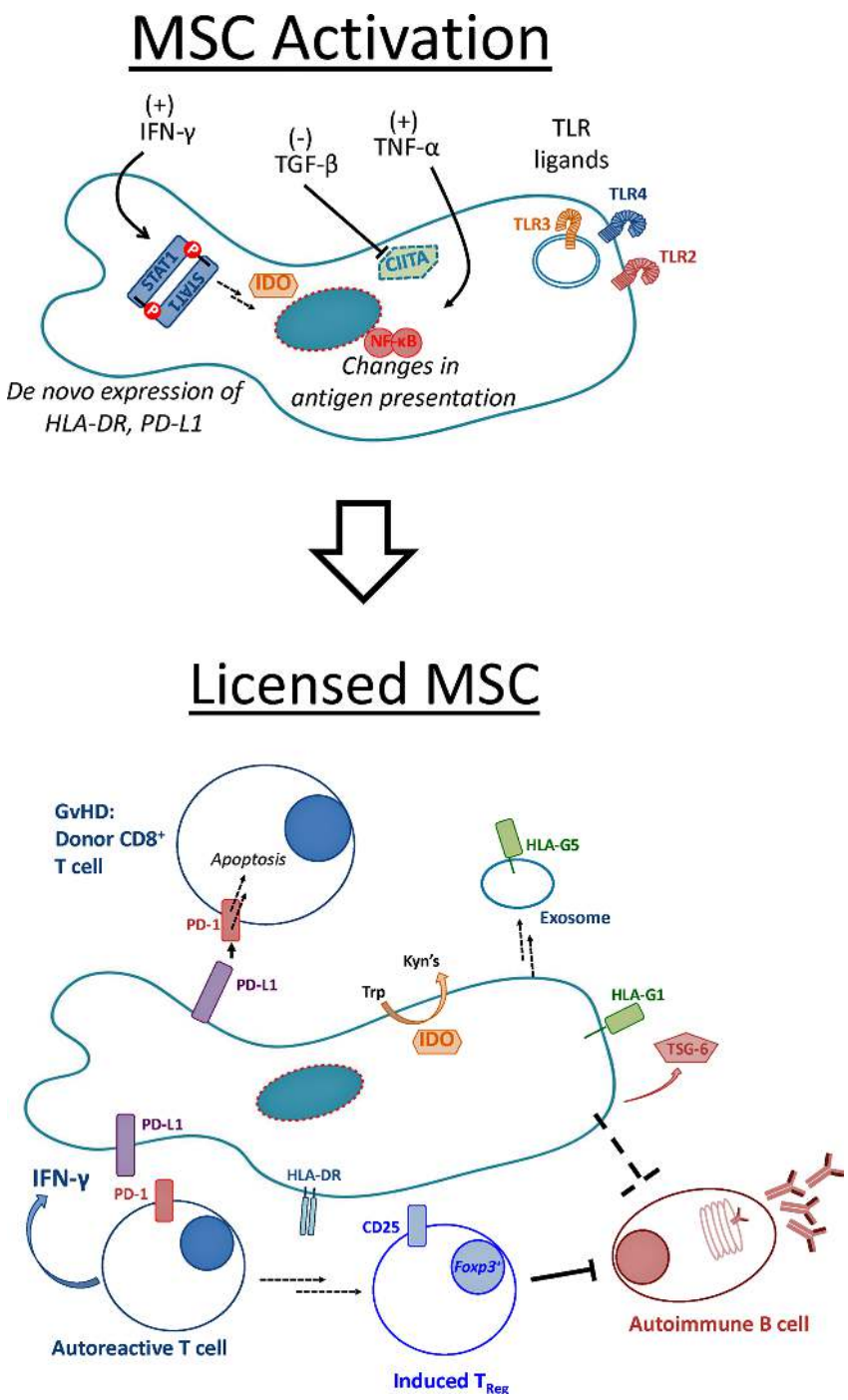



**Figure 54.8** Licensing of MSCs activates their immunomodulatory capabilities. Exposure to inflammatory stimuli causes MSCs to undergo a number of RNA- and protein-level changes, activating their immunomodulatory capabilities. This includes the *de novo* expression of PD-L1 and HLA-DR, as well as increased expression of class I HLA molecules. TNF-α signaling causes a number of NF-κB-mediated signaling events consistent with immune activation. TGF-β treatment abrogates expression of HLA-DR via inhibition of the class II transactivator CIITA [70]. After *in vitro* licensing, MSCs have been shown to more efficiently suppress the function and proliferative capacity of T and B cells. In the setting of cellular therapy it is thought that inflammatory factors in the patient can activate these same mechanisms, resulting in immunomodulation by the transfused MSCs.

However, some of these heterogeneous results may in fact be explained by the MSCs themselves. In a tissue culture system MSCs typically adopt a fibroblast-like morphology, but as culture conditions change, differences can be perceived. At low culture densities MSCs exhibit a small, round morphology characterized by rapid self-renewal. In this state MSCs express surface proteins that maintain the mesenchymal state, thereby promoting motility and inhibiting cell adhesion. After continued culture towards confluence, the cells adopt an extended fibrous shape while some of their mesenchymal plasticity and surface antigen expression is lost (see Chapter 7). This bimodal distribution of MSCs is referred to as Type 1 or Type 2 MSCs (MSC1 and MSC2 respectively). MSC1s are the smaller, self-renewing, and robustly pluripotent cells. MSC2s have reduced proliferative capacity and may be less desirable as a cellular therapeutic agent [20].

If MSC1 and MSC2 represent different temporal states of MSC populations *in vitro*, it may help to explain the differential behavior of MSCs on infusion into human patients. In the early

phases of an inflammatory insult, tissue-resident MSCs can behave like classical APCs. In response to acute-phase stimuli such as IL-1, TNF-α, or TLR agonists, MSCs upregulate HLA molecules to coordinate with the influx of first-responder leukocytes. MSCs have been shown *in vitro* to present both soluble and intracellular antigens on class I and class II molecules, in addition to cross-presentation, just as classically activated dendritic cells do [107–109]. Whether unmanipulated MSCs are capable of upregulating CCR7, and thereby trafficking to secondary lymphoid organs, remains controversial [110–112]. Nonetheless, their antigen-presenting role in the early inflammatory response plays an important role in tissue maintenance.

As inflammatory stimuli persist, the ingress of activated T cells, particularly IFN-γ-releasing Th1 cells, alter the stromal tissue cytokine milieu in which leukocytes and MSCs interact. Th1 cells are known to be pathologic in a number of chronic inflammatory conditions, and have been associated with aberrant wound repair and immune activation, as observed in sarcoidosis and cutaneous ulcers [113,114]. (Sarcoidosis is a disease involving abnormal collections of inflammatory cells (granulomas) that can form as nodules in multiple organs. The granulomas are most often located in the lungs or its associated lymph nodes, but any organ can be affected. Granulomatous inflammation is characterized primarily by accumulation of monocytes, macrophages, and activated T cells, with increased production of key inflammatory mediators such TNF, IFN-γ, IL-2, IL-8, IL-10, IL-12, IL-18, IL-23, and TGF-β, indicative of a Th1-mediated immune response.)

As discussed earlier, increasing levels of IFN-γ act on MSCs in a STAT1-dependent fashion, upregulating the enzyme IDO, the cell surface marker PD-L1, and other changes. Poised as stroma-resident immune coordinators, MSCs may integrate the signals initiated by inflammatory products, and, in physiologic conditions, tip the local balance back toward resolution and repair. Immunosuppression, so often demonstrated through *in vitro* assays, may play a crucial role *in vivo* by dampening the proliferative capacity of pathologic T cells (IDO-catalyzed changes), contact-dependent deletion (PD-L1), and the paracrine release of factors such as TSG-6, prostaglandin $E_2$, and antagonistic matrix metalloproteinase-processed CCL2 with anti-inflammatory properties.

## 54.6 Clinical applications of mesenchymal stromal cells for immune-mediated diseases

### 54.6.1 How *in vitro* data inform assessment of clinical efficacy

Discussion of the mechanistic cellular biology of MSCs is an important endeavor, for any efforts aimed at future development of these cells as a therapeutic agent must be based on scientific data. The studies described heretofore posit elegant models that seek to define the *modus operandi* for MSCs. We now turn our attention to recently generated data from larger scale clinical trials examining the therapeutic utility of MSCs in immune ailments sponsored by governmental and industry-based organizations in a variety of settings worldwide. We frame our discussion by the general platform by which clinical researchers are deploying the MSC product, and analyze these results vis-à-vis the putative mechanisms by which they are thought to act. It is our hope the reader may thereby gain a contextual framework to better understand the biology of these immunomodulatory cells.

### 54.6.2 Random donor, industrial scale

Osiris Therapeutics Inc. is a company based in Columbia, Maryland, USA, and their human bone-marrow-derived MSC product named Prochymal has been explored in a variety of clinical studies. Though much of their preclinical data have been encouraging, a recent phase III randomized placebo-controlled study failed to meet its primary endpoint in a study of severe acute graft-versus-host disease (GVHD) occurring after HSC transplantation (HSCT) [115]. In this study, overall 100-day response rate of patients receiving MSCs was 82% compared with 73% in the placebo group. Given other encouraging results using MSCs for immune-mediated diseases (notably from trials discussed later), it bears exploring some of the variations that may cause their product to achieve suboptimal results [106].

Recognizing that the development of autologous, or patient-derived, cells can sometimes be a challenge to generate in large-scale doses for patients in geographically diverse clinical trials, Osiris had sought to employ instead a so-called universal, or random donor, marrow source for their MSC product. According to the company's website and other papers published by the group [116], Osiris used MSCs from four different human sources, isolated, expanded, and archived according to good manufacturing practices as mandated by the US Food and Drug Administration for the handling of cell-based therapeutics. Many individualized dose units were then cryopreserved and shipped to health-care institutions participating in clinical trials using the Prochymal product. Given our earlier discussions regarding the differential behavior of MSCs in different cell niches, and their temporal abilities to respond to environmental cues, such a platform may be disadvantageous to clinical success. Using a massively expanded universal lot of cells could bias the overall population toward a clonal homogeneity that poorly reflects their *in vivo* niche. Clinical response in patients may therefore be blunted by infusion of suboptimal cells.

Lastly, and perhaps most importantly, for convenience of product distribution, Prochymal is shipped as a frozen bag and infused as a just-thawed product into patients. In their methods description [116], the study authors stated the cells are ascertained to have 70% viability, presumably by Trypan blue exclusion, though this was not specified. It remains an open question whether viability is the best determinant of MSC immunomodulatory abilities. This has been borne out in studies by our group and others showing that cryopreservation and

immediate infusion of thawed cells can cause dramatic loss of immune veto function [117–119].

Other studies have continued to explore the use of third-party MSCs in which the MSC donor is unrelated to, and MHC unmatched with, the MSC recipient for prophylaxis as well as treatment in GVHD occurring after HSCT. However, literature discrepancies exist regarding the definitions of response after treatment, including varied time-point assessment intervals, as well as different pharmacotherapy regimens before, after, and during MSC infusion, as well as patient age and severity of GVHD. GVHD is appreciated to be a complex syndrome, and each of the variables just mentioned can drastically alter the immunologic environment into which the MSCs are infused [120]. As perspective, the earlier described cellular biology research explored MSC function and effect through *in vitro* or animal models, but without addressing patient comorbidities or concomitant immunomodulatory therapy. Owing to variable GVHD presentations and cellular processing, it is difficult to truly compare large meta-analyses of MSC therapies [121]. A different approach may be to examine the results of such studies in consideration of their contextual framework.

One illustrative example comes from a European group in 2013 who treated a cohort of 37 children with severe grade III–IV GVHD using HLA-mismatched MSCs from cryopreserved third-party donor stocks [122]. These children had developed acute GVHD following HSCT and were treated with three doses of MSCs. Of these patients, 69% demonstrated a complete response, with the majority alive and well after a 3-year median follow-up period. The aforementioned review [121] by Introna and Rambaldi analyzes the recent developments in using MSCs immediately after HSCT as prophylaxis for GVHD, again in conjunction with standard pharmacotherapy regimens. One such study from a Russian group in 2012 infused MSCs sourced from the same donor as the transplanted HSCs as prophylaxis against GVHD [123]. Their cellular formulations are of interest. Some MSC doses were delivered fresh, others were given as thawed cellular preparations, but all cells were culture-expanded in human platelet lysate rather than fetal calf serum. Theirs was a randomized prospective study of 37 patients, mostly adults diagnosed with hematologic malignancies. The researchers demonstrate both safety and efficacy for prophylaxis, with 5% of the MSC-therapy group developing acute GVHD compared with 38% of the control group.

### 54.6.3 Allogeneic mesenchymal stromal cells, low passage

The function of MSCs in maintaining the hematopoietic niche in a healthy state and the multilevel signaling that maintains central tolerance was described mechanistically earlier. It is this very attribute of MSCs, their tolerance-enforcing role, that has motivated their application in a number of autoimmune diseases. Systemic lupus erythematosus is a complex disease with diverse symptomatology and prominent pathologic signs that imperil the gut, skin, kidneys, and nervous system (Figure 54.9).

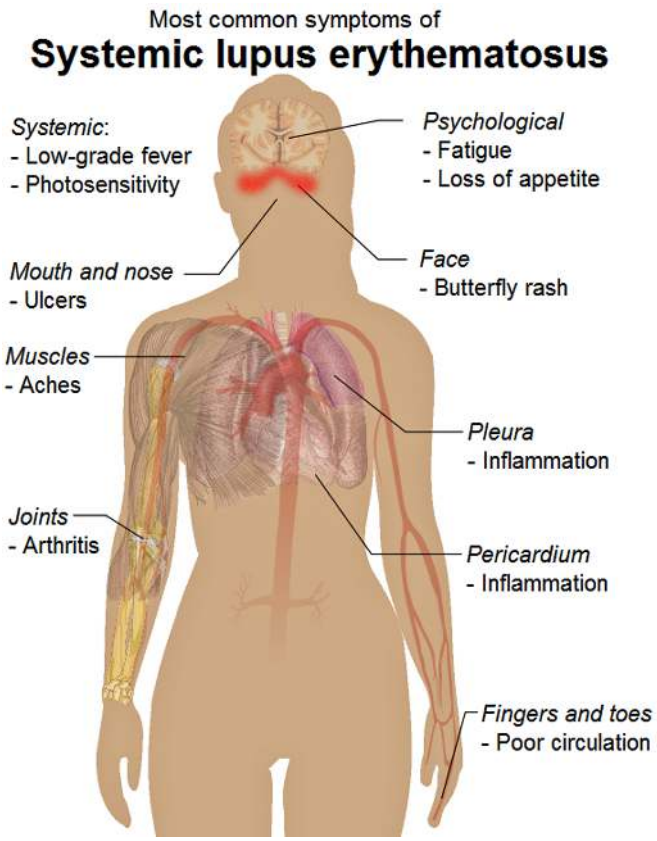



**Figure 54.9** The common symptoms of systemic lupus erythematosus, published in MedicineNet: Systemic Lupus (cont.). Last editorial review: 2009-01-30. *Source:* http://commons.wikimedia.org/wiki/File:Symptoms_of_SLE.png#/media/File:Symptoms_of_SLE.png.

Although not found in every case, antibodies that react with self-antigens have been shown to be critical to its pathogenesis. Either in the periphery or the marrow, lymphocytes that react against self-tissues are not reprogrammed into death or ignorance, and instead proliferate and wreak havoc across the body [124]. Given its immune-mediated nature, a number of research teams have sought to employ MSCs as a disease-modifying therapy in lupus.

One such study is a 2010 report that sought to treat lupus patients using autologous MSCs derived from the patients' own marrow. This study failed to show significant clinical benefit despite apparent increases in Tregs [125]. Hypothesizing that a tolerogenic defect may exist in the MSCs derived from lupus patients, a different team led by Lingyun Sun instead sought to utilize allogeneic MSCs. In a 2013 paper Sun and colleagues describe the results of 4 years' worth of clinical trial data conducted at their hospital in Nanjing, treating 87 patients with severe or drug-refractory lupus [126]. In their clinical trials, MSCs were harvested from marrow or umbilical cord, culture-expanded for no more than five *in vitro* passages and infused into patients while still in a fresh living state. Standard phenotyping and differentiation assays were performed to confirm MSC identity, but invasive studies aimed at mechanistic questions were not performed in these patients. Nonetheless, their data showed induction of clinical remission and improvement in renal function, irrespective of marrow or umbilical source.

These same researchers released a study in 2014 that sought to go further in identifying the mechanistic links whereby MSCs effect clinical improvement [127]. In a series of carefully reductionist experiments, the team replicated their allogeneic MSC data using *in vitro* assays of culture of lymphocytes and/or MSCs derived from healthy donors or patients with lupus. They demonstrated that it is the $CD8^+$ compartment of T cells that produces large amounts of IFN-γ, and that this in turn results in increased expression and enzymatic activity of IDO in healthy MSCs. Conversely, lupus-patient-derived MSCs were deficient in IDO activity, a measure that is defined as IDO catalysis of tryptophan into kynurenines. By careful comparisons of *in vitro* assays with the clinical trials, such reports demonstrate how basic cellular biology can synergize with clinical medicine to further our understanding of such cell-based therapies.

### 54.6.4 Autologous mesenchymal stromal cells for autoimmune diseases

Crohn's disease (CD) is an autoimmune disease characterized by inflammatory lesions that can occur anywhere along the gastrointestinal tract from mouth to anus. Along with ulcerative colitis, these two forms of inflammatory bowel disease (IBD) cause a spectrum of painful and damaging symptoms, including enterocutaneous fistulas, intestinal bleeding episodes, diarrhea, and malnourishment [128]; and see Chapter 50. Although the precise etiology has not been confirmed, it is generally accepted that CD arises in patients with a genetic predisposition and may represent a dysregulated immune response in the gut. The naturally tolerogenic balance of immune cells, gut tissues, and commensal flora is lost in these patients, and inflammatory cytokines such as IL-6, IL-23, and TNF-α have all been identified to play a role in its pathogenesis [129]. Although IBD can be described as an autoimmune disease, frankly self-reactive T cells or B cells are not uniformly found in all patients. Nonetheless, inflammatory damage to the gastrointestinal system is a hallmark of both ulcerative colitis and CD.

The immunologic component of IBD has been appreciated for some time, and a number of disease-modifying immunotherapies have been developed that are now in clinical use. These include anti-TNF-α antibodies such as infliximab and lymphocyte-depleting drugs drawn from the realm of chemotherapy, including azathioprine. Cell-based therapies have also been explored, including the transplantation of HSCs and MSC-based approaches. The rationale by which MSC administration is thought to improve IBD lies in their profound immunosuppressive effects, which have been described in a variety of *in vitro* and animal model systems. A number of ongoing clinical trials are now exploring MSC platforms as a therapy for CD. As has been said, interpreting these clinical data and the mechanisms by which the cells are thought to act affords us an excellent perspective on how to further improve MSC-based therapies for immune-mediated diseases.

In 2010 a Dutch group led by Hommes and Duijvestein released a report in which they described results from infusing ten CD patients with autologous bone marrow-derived MSCs [130]. Hommes and colleagues harvested MSCs from the patients, cultured the cells for no more than three passages, and then cryopreserved them prior to infusion. Patients received two infusions of cells 1 week apart. One of the distinctive aspects of this study was the battery of immunologic assays that the team employed to characterize patient responses. In addition to assessing symptom scores using the Crohn's disease activity index, the trial included endoscopic examination of lesional changes, as well as biopsies to assess T cell subsets and cytokine levels present in gut tissues. Additionally, they performed *ex vivo* T cell suppression assays, using PBMCs and MSCs derived from the same set of CD patients. Their *in vitro* experiments showed that MSCs derived from CD patients were not inferior to MSCs from healthy donors in suppressing the proliferation or cytokine secretion of CD3/CD28-stimulated PBMCs. As many of the patients received simultaneous pharmacologic treatments, the team added these drugs to co-culture systems, observing an additive effect on MSC immunosuppression. Biopsies from patients after MSC infusions showed a trend toward improvement, as suggested by Treg numbers and cytokine levels in lesional areas, but none of the patients in the study entered true long-term clinical remission. This study is nonetheless an important benchmark in MSC therapy for CD, in part because of the investigative mechanisms explored by the research team. By exploring *in vivo* the mechanisms by which MSCs have been shown to act *in vitro*, further insights are sure to follow.

## 54.7 Conclusions and next steps

It is our hope that by considering the cellular biology of the MSC we may be able to better understand the mechanisms by which these cells function in the human body, and thereby better develop cell-based therapies in the years to come. Though a number of encouraging clinical studies have been undertaken in recent years, key questions remain, most importantly regarding which immunologic mechanisms are the principal determinants of MSC bioactivity. If enzymatic catalysis of tryptophan by IDO is truly essential for their therapeutic effect, as observed in *in vitro* culture systems, then where and how is IDO activity optimized prior to giving MSCs to a patient? After intravenous injection does MSC entrapment by pulmonary vasculature stymy the cells' efficacy in modulating inflammation? And if so, does homing to diseased tissues play an important role? Although we have not discussed the genetic re-engineering approach, is expression of PD-L1, HLA-G, or CCR7 necessary for MSC efficacy and, if so, a worthwhile exploration, given the apprehension that still accompanies gene therapy?

One last consideration, but certainly not the least in terms of its priority, is how to culture MSCs *ex vivo* in order to ensure that the infused dose is as therapeutically active as possible. What are the factors that could be or should be added to culture media to make MSCs more effective? Administering cellular products with novel drug targets is already a translatable therapy in personalized cellular clinics. But which factors might exist – in the patient-derived platelet lysate, or perhaps in the conditioned media from MSCs with high IDO enzymatic activity, or the cytokines secreted by autoreactive T cells – that can ensure each infused dose of MSCs is biologically primed to exert its immune modifying effects?

The clinical studies described herein are making strong headway toward understanding MSC immunomodulation, examining peripheral and tissue-resident T cell compartments in patients after MSC administration, or subjecting MSCs to rigorous immunologic and biochemical analyses prior to infusion. Without standardized cellular processing protocols and companion potency assays, it will continue to be difficult to assess meta-analyses of MSC clinical trials. The challenge in advancing this cellular product will be to marshal enough support for one particular protocol to be consistently applied in large, statistically powerful, randomized controlled trials. As a plurality of institutes worldwide continue to utilize MSC therapies for a variety of ailments, it is essential to consider all such clinical data from the grounding perspective of molecular and cell biology and how it informs understanding and development of MSC-based therapeutics.

## References


1 Anthony, B.A. and D.C. Link. Regulation of hematopoietic stem cells by bone marrow stromal cells. *Trends Immunol*, 2014;**35**(1):32–37.

2 Dalal, J., K. Gandy, and J. Domen. Role of mesenchymal stem cell therapy in Crohn's disease. *Pediatr Res*, 2012;**71**(4 Pt 2):445–451.

3 Katayama, Y., M. Battista, W.M. Kao, *et al.* Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. *Cell*, 2006;**124**(2):407–421.

4 Omatsu, Y., T. Sugiyama, H. Kohara, *et al.* The essential functions of adipo-osteogenic progenitors as the hematopoietic stem and progenitor cell niche. *Immunity*, 2010;**33**(3):387–399.

5 Méndez-Ferrer, S., T.V. Michurina, F. Ferraro, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature*, 2010;**466**(7308):829–834.

6 Greenbaum, A., Y.M. Hsu, R.B. Day, *et al.* CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature*, 2013;**495**(7440):227–230.

7 Murphy, M.B., K. Moncivais, and A.I. Caplan. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp Mol Med*, 2013;**45**:e54.

8 Kobayashi, M., Y. Bai, Y. Dong, *et al.* PRL2/PTP4A2 phosphatase is important for hematopoietic stem cell self-renewal. *Stem Cells*, 2014;**32**(7):1956–1967.

9 Keller, J.R., M. Ortiz, and F.W. Ruscetti. Steel factor (c-kit ligand) promotes the survival of hematopoietic stem/progenitor cells in the absence of cell division. *Blood*, 1994;**86**(5):1757–1764.

10 Méndez-Ferrer, S., M. Battista, and P.S. Frenette. Cooperation of $\beta_2$- and $\beta_3$-adrenergic receptors in hematopoietic progenitor cell mobilization. *Ann N Y Acad Sci*, 2010;**1192**:139–144.

11 Jaeger S., B. Fernandez, and P. Ferrier. Epigenetic aspects of lymphocyte antigen receptor gene rearrangement or "when stochasticity completes randomness". *Immunology*, 2012;**139**:141–150.

12 Kipps, T.J. The organization and structure of lymphoid tissues. In *Williams Hematology*, 8th edn, K. Kaushansky, M.A. Lichtman, E. Beutler, *et al.* (eds). McGraw-Hill: New York, NY, 2010; chapter 5.

13 Hubert, F.X., S.A. Kinkel, G.M. Davey, *et al.* Aire regulates the transfer of antigen from mTECs to dendritic cells for induction of thymic tolerance. *Blood*, 2011;**118**(9):2462–2472.

14 Liu, G., L. Wang, T. Pang, *et al.* Umbilical cord-derived mesenchymal stem cells regulate thymic epithelial cell development and function in Foxn1$^{-/-}$ mice. *Cell Mol Immunol*, 2014;**11**(3):275–284.

15 Lin, Z.B., B. Qian, Y.Z. Yang, *et al.* Isolation, characterization and cardiac differentiation of human thymus tissue derived mesenchymal stromal cells. *J Cell Biochem*, 2015;**116**(7):1205–1212.

16 Shi, L.Z., R. Wang, G. Huang, *et al.* HIF1α-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of TH17 and Treg cells. *J Exp Med*, 2011;**208**(7):1367–1376.

17 Im, K.I., M.J. Park, N. Kim, *et al.* Induction of mixed chimerism using combinatory cell-based immune modulation with mesenchymal stem cells and regulatory T Cells for solid-organ transplant tolerance. *Stem Cells Dev*, 2014;**23**(19):2364–2376.

18 Deschaseaux, F., J. Gaillard, A. Langonné, *et al.* Regulation and function of immunosuppressive molecule human leukocyte antigen G5 in human bone tissue. *FASEB J*, 2013;**27**(8):2977–2987.

19 Naji, A., N. Rouas-Freiss, A. Durrbach, *et al.* Concise review: combining human leukocyte antigen G and mesenchymal stem cells for immunosuppressant biotherapy. *Stem Cells*, 2013;**31**(11):2296–2303.

20 Stagg, J. and J. Galipeau. Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. *Curr Mol Med*, 2013;**13**:856–867.

21 Romieu-Mourez, R., D.L. Coutu, and J. Galipeau. The immune plasticity of mesenchymal stromal cells from mice and men:

concordances and discrepancies. *Front Biosci (Elite Ed)* 2012; **E4**:824–837.

22 Kuçi, S., Z. Kuçi, H. Kreyenberg, *et al.* CD271 antigen defines a subset of multipotent stromal cells with immunosuppressive and lymphohematopoietic engraftment-promoting properties. *Haematologica*, 2010;**95**(4):651–659.

23 Matsuoka, Y., R. Nakatsuka, K. Sumide, *et al.* Prospectively isolated human bone marrow cell-derived MSCs support primitive human CD34-negative hematopoietic stem cells. *Stem Cells*, 2015;**33**:1554–1565.

24 Da Silva Meirelles, L., P.C. Chagastelles, and N.B. Nardi. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci*, 2006;**119**(Pt 11):2204–2213.

25 Kwon, D.S., X. Gao, Y.B. Liu, *et al.* Treatment with bone marrow-derived stromal cells accelerates wound healing in diabetic rats. *Int Wound J*, 2008;**5**:453–463.

26 Mackenzie, T.C. and A.W. Flake. Human mesenchymal stem cells persist, demonstrate site-specific multipotential differentiation, and are present in sites of wound healing and tissue regeneration after transplantation into fetal sheep. *Blood Cells Mol Dis*, 2001;**27** (3):601–604.

27 Chen, L., E.E. Tredget, P.Y.G. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE*, 2008;**3**(4):e1886.

28 Murray, I.R., C.C. West, W.R. Hardy, *et al.* Natural history of mesenchymal stem cells, from vessel walls to culture vessels. *Cell Mol Life Sci*, 2013;**71**(8):1353–1374.

29 Zaniboni, A., C. Bernardini, M. Alessandri, *et al.* Cells derived from porcine aorta tunica media show mesenchymal stromal-like cell properties in *in vitro* culture. *Am J Physiol Cell Physiol*, 2014;**306**: C322–C333.

30 Turner, C.G., J.D. Klein, F.L. Gray, *et al.* Craniofacial repair with fetal bone grafts engineered from amniotic mesenchymal stem cells. *J Surg Res*, 2012;**178**(2):785–790.

31 Moshaverinia, A., X. Xu., C. Chen, *et al.* Application of stem cells derived from the periodontal ligament or gingival tissue sources for tendon tissue regeneration. *Biomaterials*, 2014;**35**(9):2642–2650.

32 Götherström, C,. M. Westgren, S.W. Shaw, *et al.* Pre- and postnatal transplantation of fetal mesenchymal stem cells in osteogenesis imperfecta: a two-center experience. *Stem Cells Transl Med*, 2014;**3** (2):255–264.

33 Rafei, M., J. Hsieh, S. Fortier, *et al.* Mesenchymal stromal cell derived CCL2 suppresses plasma cell immunoglobulin production via STAT3 inactivation and PAX5 induction. *Blood*, 2008;**112**: 4991–4998.

34 Corcione, A., F. Benvenuto, E. Ferretti, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood*, 2006;**107**(1):367–372.

35 Ma, L., Z. Zhou, D. Zhang, *et al.* Immunosuppressive function of mesenchymal stem cells from human umbilical cord matrix in immune thrombocytopenia patients. *Thromb Haemost*, 2012;**107** (5):937–950.

36 Diz-Küçükkaya, R., J. Chen, A. Geddis, and J.A. López. Thrombocytopenia. In *Williams Hematology*, 8th edn, K. Kaushansky, M.A. Lichtman, E. Beutler, *et al.* (eds). McGraw-Hill: New York, NY, 2010; chapter 119.

37 Roemeling-van Rhijn, M., F.K. Mensah, S.S. Korevaar, *et al.* Effects of hypoxia on the immunomodulatory properties of adipose tissue-derived mesenchymal stem cells. *Front Immunol*, 2013;**4**:203.

38 Cuerquis, J., R. Romieu-Mourez, M. François, *et al.* Human mesenchymal stromal cells transiently increase cytokine production by activated T cells before suppressing T-cell proliferation: effect of interferon-γ and tumor necrosis factor-α stimulation. *Cytotherapy*, 2014;**16**(2):191–202.

39 Chinnadurai, R., I.B. Copland, S.R. Patel, and J. Galipeau. IDO-independent suppression of T cell effector function by IFN-γ-licensed human mesenchymal stromal cells. *J Immunol*, 2014;**192**(4):1491–1501.

40 Ribeiro, A., P. Laranjeira, S. Mendes, *et al.* Mesenchymal stem cells from umbilical cord matrix, adipose tissue and bone marrow exhibit different capability to suppress peripheral blood B, natural killer and T cells. *Stem Cell Res Ther*, 2013;**4**(5):125.

41 Bocelli-Tyndall, C., L. Bracci, S. Schaeren, *et al.* Human bone marrow mesenchymal stem cells and chondrocytes promote and/or suppress the *in vitro* proliferation of lymphocytes stimulated by interleukins 2, 7 and 15. *Ann Rheum Dis*, 2009;**68**(8): 1352–1359.

42 Engela, A.U., C.C. Baan, N.H. Litjens, *et al.* Mesenchymal stem cells control alloreactive $CD8^+$ $CD28^-$ T cells. *Clin Exp Immunol*, 2013;**174**(3):449–458.

43 Singer, N.G. and A.I. Caplan. Mesenchymal stem cells: mechanisms of inflammation. *Annu Rev Pathol*, 2011;**6**:457–478.

44 Uccelli, A., L. Moretta, and V. Pistoia. Mesenchymal stem cells in health and disease. *Nat Rev Immunol*, 2008;**8**(9):726–736.

45 Le Blanc, K. and D. Mougiakakos. Multipotent mesenchymal stromal cells and the innate immune system. *Nat Rev Immunol*, 2012;**12**(5):383–396.

46 Schmid, M. and D.A. Carson. Cell-cycle regulation and hematologic disorders. In: *Williams Hematology*, 8th edn, K. Kaushansky, M.A. Lichtman, E. Beutler, *et al.* (eds). McGraw-Hill: New York, NY, 2010; chapter 13.

47 Corthay, A. Does the immune system naturally protect against cancer? *Front Immunol*, 2014;**5**:197.

48 Afshar-Sterle, S., D. Zotos, N.J. Bernard, *et al.* Fas ligand-mediated immune surveillance by T cells is essential for the control of spontaneous B cell lymphomas. *Nat Med*, 2014;**20**(3):283–290.

49 Tu, M.M., A.B. Mahmoud, A. Wight, *et al.* Ly49 family receptors are required for cancer immunosurveillance mediated by natural killer cells. *Cancer Res*, 2014;**74**(14):3684–3694.

50 Akbay, E.A., S. Koyama, J. Carretero, *et al.* Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. *Cancer Discov*, 2013;**3**(12):1355–1363.

51 Dai, S., R. Jai, X. Zhang, *et al.* The PD-1/PD-Ls pathway and autoimmune diseases. *Cell Immunol*, 2014;**290**(1):72–79.

52 Dong, H., S.E. Strome, D.R. Salomao, *et al.* Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med*, 2002;**8**(8):793–800.

53 Latchman, Y., C.R. Wood, T. Chernova, *et al.* PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol*, 2001;**2** (3):261–268.

54 Hirano, F., K. Kaneko, H. Tamura, *et al.* Blockade of B7-H1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res*, 2005;**65**:1089–1096.

55 Iwai, Y., M. Ishida, Y. Tanaka, *et al.* Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A*, 2002;**99** (19):12293–12297.

56 Sundar, R., R. Soong, B.-C. Cho, *et al.* Immunotherapy in the treatment of non-small cell lung cancer. *Lung Cancer*, 2014;**85**(2):101–109.

57 Tang, P.A. and D.Y. Heng. Programmed death 1 pathway inhibition in metastatic renal cell cancer and prostate cancer. *Curr Oncol Rep*, 2013;**15**(2):98–104.

58 Westin, J.R., F. Chu, M. Zhang, *et al.* Safety and activity of PD1 blockade by pidilizumab in combination with rituximab in patients with relapsed follicular lymphoma: a single group, open-label, phase 2 trial. *Lancet Oncol*, 2014;**15**(1):69–77.

59 US Food and Drug Administration. *FDA approves Keytruda for advanced melanoma*. FDA News Release, September 4, 2014. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm412802.htm (accessed April 8, 2016).

60 Augello, A., R. Tasso, S.M. Negrini, *et al.* Bone marrow mesenchymal progenitor cells inhibit lymphocyte proliferation by activation of the programmed death 1 pathway. *Eur J Immunol*, 2005;**35**(5):1482–1490.

61 Sheng, H., Y. Wang, Y. Jin, *et al.* A critical role of IFNγ in priming MSC-mediated suppression of T cell proliferation through up-regulation of B7-H1. *Cell Res*, 2008;**18**(8):846–857.

62 Luz-Crawford, P., D. Noël, X. Fernandez, *et al.* Mesenchymal stem cells repress Th17 molecular program through the PD-1 pathway. *PLoS ONE*, 2012;**7**(9):e45272.

63 Luz-Crawford, P., M. Kurte, J. Bravo-Alegría, *et al.* Mesenchymal stem cells generate a $CD4^+CD25^+Foxp3^+$ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem Cell Res Ther*, 2013;**4**(3):65.

64 Prendergast, G.C., C. Smith, S. Thomas, *et al.* Indoleamine 2,3-dioxygenase pathways of pathogenic inflammation and immune escape in cancer. *Cancer Immunol Immunother*, 2014;**63**(7):721–735.

65 Abumaree, M.H., L.W. Chamley, M. Badri, and M.F. El-Muzaini. Trophoblast debris modulates the expression of immune proteins in macrophages: a key to maternal tolerance of the fetal allograft? *J Reprod Immunol*, 2012;**94**(2):131–141.

66 Sucher, R., K. Fischler, R. Oberhuber, *et al.* IDO and regulatory T cell support are critical for cytotoxic T lymphocyte-associated Ag-4 Ig-mediated long-term solid organ allograft survival. *J Immunol*, 2012;**188**(1):37–46.

67 Boyland, B. and D.C. Williams. The metabolism of tryptophan: 2. The metabolism of tryptophan in patients suffering from cancer of the bladder. *Biochem J*, 1956;**64**(3):578–582.

68 Pallotta, M.T., C. Orabona, C. Volpi, *et al.* Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nat Immunol*, 2011;**12**(9):870–878.

69 Wen, L., M. Zhu, M.C. Madigan, *et al.* Immunomodulatory effects of bone marrow-derived mesenchymal stem cells on pro-inflammatory cytokine-stimulated human corneal epithelial cells. *PLoS ONE*, 2014;**9**(7):e101841.

70 DelaRosa, O., E. Lombardo, A. Beraza, *et al.* Requirement of IFN-γ-mediated indoleamine 2,3-dioxygenase expression in the modulation of lymphocyte proliferation by human adipose-derived stem cells. *Tissue Eng Part A*, 2009;**15**(10):2795–2806.

71 Croitoru-Lamoury, J., F.M.J. Lamoury, M. Caristo, *et al.* Interferon-γ regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). *PLoS ONE*, 2011;**6**(2):e14698.

72 François, M., R. Romieu-Mourez, M. Li, and J. Galipeau. Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. *Mol Ther*, 2012;**20**(1):187–195.

73 Németh, K., A. Leelahavanichkul, P.S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nat Med*, 2009;**15**(1):42–49.

74 Geng, Y., L. Zhang, B. Fu, *et al.* Mesenchymal stem cells ameliorate rhabdomyolysis-induced acute kidney injury via the activation of M2 macrophages. *Stem Cell Res Ther*, 2014;**5**(3):80.

75 Fahy, N., M.L. de Vries-van Melle, J. Lehmann, *et al.* Human osteoarthritic synovium impacts chondrogenic differentiation of mesenchymal stem cells via macrophage polarisation state. *Osteoarthritis Cartilage*, 2014;**22**(8):1167–1175.

76 Meisel, R., A. Zibert, M. Laryea, *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood*, 2004;**103**(12):4619–4621.

77 Jui H.Y., C.H. Lin, W.T. Hsu, *et al.* Autologous mesenchymal stem cells prevent transplant arteriosclerosis by enhancing local expression of interleukin-10, interferon-γ, and indoleamine 2,3-dioxygenase. *Cell Transplant*, 2012;**21**(5):971–984.

78 Ge, W., J. Jiang, J. Arp, *et al.* Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation*, 2010;**90**(12):1312–1320.

79 Warren, J.S. and P.A. Ward. The inflammatory response. In *Williams Hematology*, 8th edn, K. Kaushansky, M.A. Lichtman, E. Beutler, *et al.* (eds). McGraw-Hill: New York, NY, 2010; chapter 17.

80 Smith, L.C., K. Azumi, and M. Nonaka. Complement systems in invertebrates. The ancient alternative and lectin pathways. *Immunopharmacology*, 1999;**42**(1–3):107–120.

81 Schraufstatter, I.U., R.G. Discipio, M. Zhao, and S.K. Khaldoyanidi. C3a and C5a are chemotactic factors for human mesenchymal stem cells, which cause prolonged ERK1/2 phosphorylation. *J Immunol*, 2009;**182**(6):3827–3836.

82 Moll, G., R. Jitschin, L. von Bahr, *et al.* Mesenchymal stromal cells engage complement and complement receptor bearing innate effector cells to modulate immune responses. *PLoS ONE*, 2011;**6**(7):e21703.

83 Conte, M.P., C. Longhi, M. Marazzato, *et al.* Adherent-invasive *Escherichia coli* (AIEC) in pediatric Crohn's disease patients: phenotypic and genetic pathogenic features. *BMC Res Notes*, 2014;**7**:748.

84 Van den Akker, F., S.C. de Jager, and J. P. Sluijter. Mesenchymal stem cell therapy for cardiac inflammation: immunomodulatory properties and the influence of toll-like receptors. *Mediators Inflamm*, 2013;**2013**:181020.

85 Delarosa, O., W. Dalemans, and E. Lombardo. Toll-like receptors as modulators of mesenchymal stem cells. *Front Immunol*, 2012;**3**:182.

86 Liotta, F., R. Angeli, L. Cosmi, *et al.* Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells*, 2008;**26**(1):279–289.

87 Romieu-Mourez, R., M. François, M.N. Boivin, *et al.* Cytokine modulation of TLR expression and activation in mesenchymal

stromal cells leads to a proinflammatory phenotype. *J Immunol*, 2009;**182**(12):7963–7973.

88 Mosmann, T.R., H. Cherwinski, M.W. Bond, *et al.* Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol*, 1986;**136**(7):2348–2357.

89 Krampera, M., L. Cosmi, R. Angeli, *et al.* Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells*, 2006;**24**(2):386–398.

90 Ryan, J.M., F. Barry, J.M. Murphy, and B. P. Mahon. Interferon-γ does not break, but promotes the immunosuppressive capacity of adult human mesenchymal stem cells. *Clin Exp Immunol*, 2007;**149**(2):353–363.

91 Romieu-Mourez, R., M. François, M.-N. Boivin, *et al.* Regulation of MHC class II expression and antigen processing in murine and human mesenchymal stromal cells by IFN-γ, TGF-β, and cell density. *J Immunol*, 2007;**179**(3):1549–1558.

92 Beutler B,. D. Greenwald, J.D. Hulmes, *et al.* Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature*, 1985;**316**(6028):552–554.

93 Piao, J.H., H. Yoshida, W.C. Yeh, *et al.* TNF receptor-associated factor 2-dependent canonical pathway is crucial for the development of Peyer's patches. *J Immunol*, 2007;**178**(4):2272–2277.

94 Furtado, G.C., M.E. Pacers, G. Bongers, *et al.* TNFα-dependent development of lymphoid tissue in the absence of RORγt$^+$ lymphoid tissue inducer cells. *Mucosal Immunol*, 2014;**7**(3):602–614.

95 Kaushansky, K. Signal transduction pathways. In: *Williams Hematology*, 8th edn, K. Kaushansky, M.A. Lichtman, E. Beutler, *et al.* (eds). McGraw-Hill: New York, NY, 2010; chapter 14.

96 Dorronsoro, A., I. Ferrin, J.M. Salcedo, *et al.* Human mesenchymal stromal cells modulate T-cell responses through TNF-α-mediated activation of NF-κB. *Eur J Immunol*, 2014;**44**(2):480–488.

97 Dorronsoro A., Lang V., Jakobsson E., *et al.* Identification of the NF-κB inhibitor A20 as a key regulator for human adipogenesis. *Cell Death Dis* 2013;**4**:e972.

98 Jansen, A.H., E.A. Reits, and E.M. Hol. The ubiquitin proteasome system in glia and its role in neurodegenerative diseases. *Front Mol Neurosci*, 2014;**7**:73.

99 Shen, M., S. Schmitt, D. Buac, and Q. P. Dou., Targeting the ubiquitin–proteasome system for cancer therapy. *Expert Opin Ther Targets*, 2013;**17**(9):1091–1108.

100 Song, X.T., K. Evel-Kabler, L. Shen, *et al.* A20 is an antigen presentation attenuator, and its inhibition overcomes regulatory T cell-mediated suppression. *Nat Med*, 2008;**14**(3):258–265.

101 Lee, T.H., H.G. Wisniewski, and J. Vilcek. A novel secretory tumor necrosis factor-inducible protein (TSG-6) is a member of the family of hyaluronate binding proteins, closely related to the adhesion receptor CD44. *J Cell Biol*, 1992;**116**(2):545–557.

102 Lee, R.H., A.A. Pulin, M.J. Seo, *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell*, 2009;**5**(1):54–63.

103 Choi, H., R.H. Lee, N. Bazhanov, *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood*, 2011;**118**(2):330–338.

104 Ren, G., L. Zhang, X. Zhao, *et al.* Mesenchymal stem cell-mediated immunosuppression occurs via concerted action of chemokines and nitric oxide. *Cell Stem Cell*, 2008;**2**(2):141–150.

105 Romieu-Mourez, R., M. François, A. Abate, *et al.* Mesenchymal stromal cells expressing ErbB-2/neu elicit protective antibreast tumor immunity *in vivo,* which is paradoxically suppressed by IFN-γ and tumor necrosis factor-α priming. *Cancer Res*, 2010;**70**(20):7742–7747.

106 Galipeau, J. The mesenchymal stromal cells dilemma – does a negative phase III trial of random donor mesenchymal stromal cells in steroid-resistant graft-versus-host disease represent a death knell or a bump in the road? *Cytotherapy*, 2013;**15**(1):2–8.

107 François, M., R. Romieu-Mourez, S. Stock-Martineau, *et al.* Mesenchymal stromal cells cross-present soluble exogenous antigens as part of their antigen-presenting cell properties. *Blood*, 2009;**114**(13):2632–2638.

108 Najar, M., G. Raicevic, H. Fayyad-Kazan, *et al.* Immune-related antigens, surface molecules and regulatory factors in human-derived mesenchymal stromal cells: the expression and impact of inflammatory priming. *Stem Cell Rev*, 2012;**8**(4):1188–1198.

109 Raicevic, G., M. Najar, M. Najimi, *et al.* Influence of inflammation on the immunological profile of adult-derived human liver mesenchymal stromal cells and stellate cells. *Cytotherapy*, 2015;**17**(2):174–185.

110 Li, H., Y. Jiang, X. Jiang, *et al.* CCR7 guides migration of mesenchymal stem cell to secondary lymphoid organs: a novel approach to separate GvHD from GvL effect. *Stem Cells*, 2014;**32**(7):1890–1903.

111 Brooke, G., H. Tong, J.P. Levesque, and K. Atkinson. Molecular trafficking mechanisms of multipotent mesenchymal stem cells derived from human bone marrow and placenta. *Stem Cells Dev*, 2008;**17**(5):929–940.

112 Li, H., Y.M. Jiang, Y.F. Sun, *et al.* CCR7 expressing mesenchymal stem cells potently inhibit graft-versus-host disease by spoiling the fourth supplemental Billingham's tenet. *PLoS ONE*, 2014;**9**(12):e115720.

113 Ringkowski, S., P.S. Thomas, and C. Herbert. Interleukin-12 family cytokines and sarcoidosis. *Front Pharmacol*, 2014;**5**:233.

114 Barros, F.M., M.A. Lotufo, P.M. Andrade, *et al.* Possible association between Th1 immune polarization and epithelial permeability with toll-like receptors 2 dysfunction in the pathogenesis of the recurrent aphthous ulceration. *Ulcers*, 2010;**2010**:163804.

115 Martin, P.J., J.P. Uberti, R.J. Soiffer, *et al.* Prochymal improves response rates in patients with steroid-refractory acute graft versus host disease (SR-GVHD) involving the liver and gut: Results of a randomized, placebo-controlled, multicenter phase III trial in GVHD. *Biol Blood Marrow Transplant*, 2010;**16**(2, Suppl 2):S169–S170.

116 Prasad, V.K., K.G. Lucas, G.I. Kleiner, *et al.* Efficacy and safety of *ex vivo* cultured adult human mesenchymal stem cells (Prochymal) in pediatric patients with severe refractory acute graft-versus-host disease in a compassionate use study. *Biol Blood Marrow Transplant*, 2011;**17**(4):534–541.

117 Ragoonanan, V., A. Hubel, and A. Aksan. Response of the cell membrane-cytoskeleton complex to osmotic and freeze/thaw stresses. *Cryobiology*, 2010;**61**(3):335–344.

118 Castelo-Branco MT., I.D. Soares, D.V. Lopes, *et al.* Intraperitoneal but not intravenous cryopreserved mesenchymal stromal cells

home to the inflamed colon and ameliorate experimental colitis. *PLoS ONE*, 2012;**7**(3):e33360.

119 Chinnadurai, R., M.A. Garcia, Y. Sakurai, *et al.* Actin cytoskeletal disruption following cryopreservation alters the biodistribution of human mesenchymal stromal cells *in vivo*. *Stem Cell Rep*, 2014;**3** (1):60–72.

120 Kim, N., K.I. Im, J.Y. Lim, *et al.* Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: experiments and practice. *Ann Hematol*, 2013;**92**(10):1295–1308.

121 Introna, M. and A. Rambaldi. Mesenchymal stromal cells for prevention and treatment of graft-versus-host disease: successes and hurdles. *Curr Opin Organ Transplant*, 2015;**20**(1): 72–78.

122 Ball, L.M., M.E. Bernardo, H. Roelofs, *et al.* Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III–IV acute graft-versus-host disease. *Br J Haematol*, 2013;**163**(4):501–509.

123 Kuzmina, L.A., N.A. Petinati, E.N. Parovichnikova, *et al.* Multipotent mesenchymal stromal cells for the prophylaxis of acute graft-versus-host disease – a phase II study. *Stem Cells Int*, 2012;**2012**:968213.

124 Hahn, B.H. Systemic lupus erythematosus. In: *Harrison's Principles of Internal Medicine*, 18th edn, D.L. Longo, S. Fauci, D. L. Kasper, *et al.* (eds). McGraw-Hill: New York, NY, 2012; chapter 319.

125 Carrion, F., E. Nova, C. Ruiz, *et al.* Autologous mesenchymal stem cell treatment increased T regulatory cells with no effect on disease activity in two systemic lupus erythematosus patients. *Lupus*, 2010;**19**:317–322.

126 Wang, D., H. Zhang, J. Liang, *et al.* Allogeneic mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus: 4 years of experience. *Cell Transplant*, 2013;**22**:2267–2277.

127 Wang, D., X. Feng, L. Lu, *et al.* A CD8 T cell/indoleamine 2,3-dioxygenase axis is required for mesenchymal stem cell suppression of human systemic lupus erythematosus. *Arthritis Rheumatol*, 2014;**66**(8):2234–2245.

128 Friedman, S. and R.S. Blumberg. Inflammatory bowel disease. In: *Harrison's Principles of Internal Medicine*, 18th edn, D.L. Longo, S. Fauci, D. L. Kasper, *et al.* (eds). McGraw-Hill: New York, NY, 2012; chapter 295.

129 Blumberg, R.S. and S.B. Snapper. Inflammatory bowel disease: immunologic considerations & therapeutic implications. In: *Current Diagnosis & Treatment: Gastroenterology, Hepatology, & Endoscopy*, 2nd edn, N.J. Greenberger, R.S. Blumberg, and R. Burakoff (eds). McGraw-Hill: New York, NY, 2012; chapter 2.

130 Duijvestein, M., A.C. Vos, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cell treatment for refractory luminal Crohn's disease: results of a phase I study. *Gut*, 2010;**59** (12):1662–1669.

CHAPTER 55

# The role of mesenchymal stromal cells in bacterial infection

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## 55.1 Introduction

Sepsis is a disease process representing the systemic response to severe infection. It is characterized by dysregulation of the host immune and inflammatory mechanisms [1]. The majority of cases of sepsis are due to bacterial infections. As a result, bacterial infections and sepsis represent a significant cause of mortality and morbidity [2]. The infectious insult activates the immune cells of the host, including neutrophils, monocytes/macrophages, and lymphocytes. This early activation of immune cells triggers a proinflammatory response that contributes to the eradication of the invading microorganisms [3]. Systemically, elevated levels of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 have been described in sepsis [4]. If the pathogens are not eradicated then sepsis can progress in severity with associated organ dysfunction and ultimately death. Even if the invading microorganism is eradicated the early proinflammatory response may also be followed by a later stage of immune paralysis, due in part to apoptosis of immune effector cells [5]. Despite an improved understanding of sepsis and its management, it still remains a significant and complex clinical problem. As a result, new and innovative treatment strategies are necessary to target each stage of sepsis [6].

One of the treatment strategies currently being investigated is the use of mesenchymal stromal cells (MSCs) for bacterial infections and sepsis [7]. MSCs are considered to be a promising platform for cell-based therapy. They possess a variety of anti-inflammatory and cytoprotective properties that could regulate the process of sepsis-induced inflammation and organ injury via several mechanisms targeting the various stages of sepsis [8]. MSCs express low levels of major histocompatibility complex (MHC) class I molecules but do not express MHC class II or costimulatory molecules such as CD80, CD86, and CD40. Therefore, they are considered to be immune-privileged cells and can be successfully transplanted across allogeneic barriers [9]. This chapter will review the properties of MSCs that can be used to target the harmful effects of bacterial infections as well as the data obtained from the use of MSCs in preclinical animal models of sepsis and the proposed mechanisms behind these findings. The proposed beneficial properties of MSCs against bacterial infection and sepsis are summarized in Figure 55.1.

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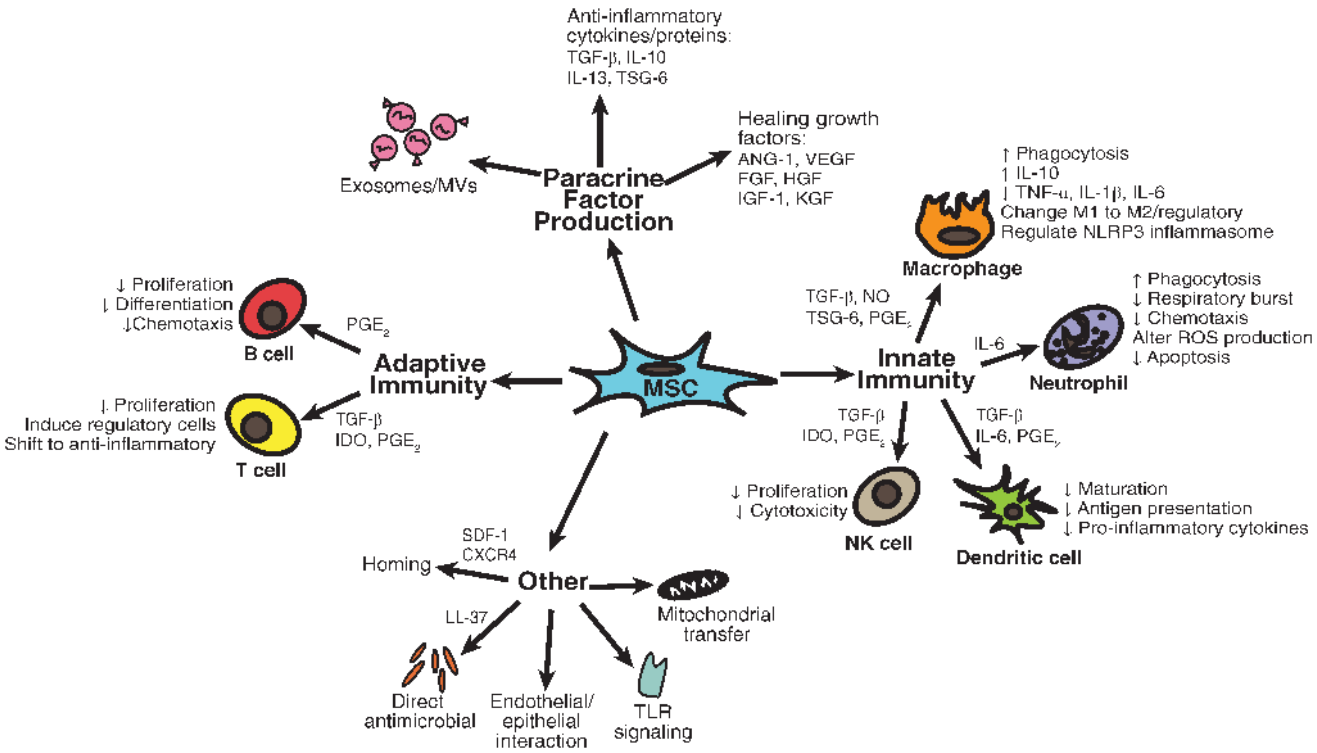



**Figure 55.1** Proposed effects of MSCs in response to bacterial infection. ANG-1: angiopoietin-1; CXCR4: C—X—C chemokine receptor 4; FGF: fibroblast growth factor; HGF: hepatocyte growth factor; IDO: inoleamine 2,3-dioxygenase; IGF: insulin-like growth factor; KGF: keratinocyte growth factor; LL-37: human cathelicidin (also known as human cationic antimicrobial protein 18); MVs: microvesicles; NK: natural killer; NLRP3: NOD-like receptor family, pyrin domain containing 3; NO: nitric oxide; $PGE_2$: prostaglandin $E_2$; ROS: reactive oxygen species; SDF: stromal-cell-derived factor; TGF: transforming growth factor; TSG: TNF-α-stimulated gene/protein; TLR: toll-like receptor; VEGF: vascular endothelial growth factor.

## 55.2 Experimental models of bacterial infection and sepsis

MSCs have been tested in a variety of preclinical models of bacterial infection and sepsis. The complexity of these disease processes makes the clinical study of potential therapeutics difficult. As a result, animal models have been developed to help understand the pathogenesis of sepsis and to trial potential therapies such as MSCs. Sepsis models can be divided into three categories: exogenous administration of a toxin, exogenous administration of bacteria, or alteration of the animal's endogenous protective barrier [10]. As these models try to mimic the multistage picture of clinical sepsis, they each have their own limitations.

The lipopolysaccharide (LPS) or endotoxin model is an example of the exogenous administration of a toxin. LPS is the glycolipid that is present in the outer membrane of all Gram-negative bacteria. (Gram-staining was developed by Hans Christian Joachim Gram, a Danish bacteriologist, in 1884.) Exogenous administration of LPS causes a sepsis-like picture characterized by an elevated level of circulating proinflammatory cytokines without bacteremia [11]. This model is simple to perform and widely used. LPS has been given via a variety of routes and used in several species, including an *ex vivo* perfused human lung [12]. The LPS model has been criticized in that a bolus injection of LPS results in a rapid but transient host response that differs from the prolonged response of human disease [10]. Formylated peptides are a group of bacteria-derived components that are used to induce infection in preclinical models [13].

Animals can also be inoculated directly with bacteria. These models can be tailored by altering the route of infection; for example, into the blood, intraperitoneally, subcutaneously, or into the lung. The frequency of administration and the mode of administration, such as a bolus injection, a continuous infusion, or by an osmotic pump, can also be selected, as can the bacterial strain and the size of the inoculum. All of these parameters can affect progression and outcome. Direct inoculation has been used in a variety of animals, including mice, rats, and sheep [14,15].

The fibrin clot model is a variation of the direct inoculation model. It introduces a strain of bacteria into the peritoneal cavity and ultimately the bloodstream. It consists of entrapping a set dose of bacteria into a fibrin clot and performing a midline laparotomy to implant the fibrin clot into the peritoneal cavity. The benefits of this model are that it allows a slower release of bacteria and a more sustained infection [16]. Alternatives to a fibrin clot, such as a gelatin capsule, have also been used to introduce bacteria into the peritoneal cavity [17].

Host-barrier disruption models of sepsis involve disrupting the normal protective barriers that shield sterile compartments from pathogenesis. Cecal ligation and puncture (CLP) is an example of this type of model. It involves a midline laparotomy followed by externalizing, ligating, and puncturing the cecum. The perforated cecum then serves to leak fecal contents into the peritoneum, followed later by translocation of enteric bacteria into the blood. This establishes a polymicrobial model of infection as well as an inflammatory source of necrotic tissue. The amount of cecum ligated and the number and size of punctures can control the severity of the model [18]. The CLP technique is commonly employed owing to its ease of use. While the model is thought to be reproducible, there is operator variability as well as variability introduced from the amount of the load of fecal material in the ligated cecum. Despite its limitations, CLP is currently the most widely used animal model of sepsis [19].

The colon ascendens stent peritonitis is another example of a host-barrier disruption model of sepsis. It consists of a defined-diameter stent being placed into the ascending colon of mice, leading to persistent leakage of fecal content into the peritoneal cavity. Stent diameter is used to control the severity of disease induced. The stent can also be removed after defined time periods. Like CLP, it is also a model of acute polymicrobial septic peritonitis characterized by a rise in proinflammatory cytokines. However, systemic cytokine levels and bacterial blood counts are thought to be greater than in the CLP model. The colon ascendens stent peritonitis model has had limited usage as it involves a more challenging surgical procedure than CLP. MSCs have not yet been trialed in this model [10].

In order to develop a more clinically relevant model of sepsis, researchers have employed "two-hit" models. These models seek to mimic the changes in the host immunologic response with disease progression. For example, CLP is typically used as the "first hit" followed by bacterial pneumonia as the "second hit." This strategy seeks to encompass the early proinflammatory phase and the later state of immune paralysis [20].

All preclinical models differ from human sepsis because of age, comorbidity, and use of supportive therapy [19]. Despite these limitations, preclinical models have been a very important tool for studying the effects of MSCs on infection and sepsis. Bone marrow remains the most common source of MSCs. However, in recent years other sources of MSCs, such as compact bone and adipose-tissue-derived (AD)-MSCs, have shown similar effects [21]. Adipose-derived MSCs show promise, and they are much more readily available than bone-marrow-derived (BM)-MSCs [22]. MSCs can be given at various time points before, during, and after the infectious insult in all of these models. Their administration has conferred a survival benefit in many of these models. In addition to improving mortality, they also have been shown to have many other effects on the septic picture [23]. The remainder of this chapter will explore the potential mechanisms by which MSCs can improve outcomes in bacterial infections and sepsis. The findings from preclinical models are summarized in Table 55.1.

## 55.3 Effects of mesenchymal stromal cells on the innate immune response

In sepsis, patients surviving the initial infectious insult can develop features of immunosuppression that can result in secondary infection, organ dysfunction, and death. This immunosuppressive state is caused in part by apoptotic immune cell loss and immune cell dysfunction that occurs by a chronically excessive proinflammatory state [8]. MSCs interact with the innate immune system in several ways that may be beneficial in modulating this detrimental inflammatory response (and see Chapter 33).

Neutrophils are one of the important cells of the innate immune system and are considered a first line of defense against pathogens. These cells are rapidly mobilized in the setting of bacterial infections and are activated to kill microorganisms via a process known as the respiratory burst that occurs during phagocytosis. The respiratory burst (sometimes called the oxidative burst) is the rapid release of ROS (superoxide radical and hydrogen peroxide) from different types of cells. While neutrophils are critical for the clearance of bacteria, they can also result in collateral injury of normal tissue. MSCs have been shown to dampen the respiratory burst. In addition, MSCs have also been shown to inhibit neutrophil chemotaxis [26].

It has also been demonstrated that MSCs may promote the generation of ROS in neutrophils under direct *E. coli* stimulation to eliminate invading microbes. However, in the presence of MSCs, formylated-peptide-stimulated neutrophils demonstrated a decrease in ROS production [39,40]. These different outcomes may have been caused by different stimulators of the respiratory burst (e.g., live bacteria as opposed to bacteria-derived components), different incubation approaches, or the use of conditioned media (CM) instead of cells. In the former of these reports neutrophils were treated with CM, whereas in the latter report MSCs were co-cultured with neutrophils.

A harmful effect of excessive inflammation during sepsis is the apoptotic death of immune cells by activation of the caspase cascade. MSCs have been shown to delay the spontaneous apoptosis of resting and activated neutrophils through an IL-6-dependent mechanism [40]. This finding was seen in mice after CLP, in which MSCs were able to prevent apoptotic cell death in the lung and kidneys [25].

Finally, the importance of the MSC and neutrophil interaction in bacterial infections was demonstrated in a CLP-induced polymicrobial sepsis model in mice. Hall *et al.* demonstrated that the therapeutic effect of MSCs was dependent on endogenous neutrophils. In particular, MSC treatment failed to rescue neutropenic animals from CLP-induced septic death compared with normal mice. These investigators also found that MSCs may enhance bacterial phagocytosis in neutrophils in a significant manner. This phagocytic enhancement led to a further decrease in bacterial burden [36].

Macrophages and monocytes are another type of immune cell thought to play an important role in phagocytosis of invading

**Table 55.1** Publications on the use of MSCs in preclinical models of infection and sepsis.

| Model | Species | MSC methods | MSC effects | Reference |
|---|---|---|---|---|
| *Exogenous toxin* | | | | |
| *Escherichia coli* endotoxin ALI | Mouse | $7.5 \times 10^5$ mouse BM-MSCs, IT, 4 h post ALI | Increased survival<br>Decrease in excess lung water and BAL protein<br>Decreased proinflammatory cytokines and increased IL-10 | [24] |
| LPS-induced ALI | Mouse | $2 \times 10^5$ mouse BM-MSCs overexpressing ANG-1, IV, 30 min post LPS | Improvement in alveolar inflammation and permeability | [25] |
| Endotoxemia/LPS | Mouse | $5 \times 10^5$ mouse BM-MSCs, IV 1 h postLPS | Decreased proinflammatory cytokines | [26] |
| Endotoxemia/LPS | Mouse | $1 \times 10^5$–$10^6$ human AD-MSCs, IP, 30 min post LPS | Improved survival<br>Decreased proinflammatory cytokines and increased IL-10<br>Reduced inflammatory cell organ infiltration | [22] |
| *E. coli* endotoxin ALI | *Ex vivo* perfused human lung | $5 \times 10^6$ human BM-MSCs or MSC-CM, IB, 1 h post ALI | Decreased extravascular lung water<br>Improved lung endothelial barrier permeability<br>Restored alveolar fluid clearance<br>Reduced inflammatory cell lung infiltration and septal thickening | [12] |
| Endotoxemia/LPS | Rat | $2 \times 10^6$ mouse BM-MSCs IP, 1 h post LPS | Reduced proinflammatory cytokines in blood and myocardium<br>Increased IL-10<br>Attenuated myocardial functional depression | [27] |
| *E. coli* endotoxin ALI | Mouse | 30 μL MVs (from $3 \times 10^6$ human BM-MSCs), IT, with ALI | Decreased extravascular lung water<br>Reduced total protein in BAL fluid<br>Decreased lung inflammation<br>Reduced influx of neutrophils in lung | [28] |
| Staphylococcal enterotoxin B TSS | Mouse | $2 \times 10^5$ human BM-MSCs, IV, 1 h before TSS | Decreased proinflammatory cytokines<br>No improvement on survival | [29] |
| *Exogenous bacteria* | | | | |
| *E. coli* pneumonia | Mouse | $1 \times 10^6$ human BM-MSCs IT, 4 h post pneumonia | Reduced bacterial growth in lung and BAL fluid<br>Effects mediated by secretion of antimicrobial peptide LL-37 | [30] |
| *Pseudomonas aeruginosa* peritonitis | Mouse | $1 \times 10^6$ human BM-MSCs, IV, 1 h post bacteria | Improved survival<br>Decreased bacteremia<br>Enhanced phagocytosis by monocytes | [31] |
| *E. coli* pneumonia/ALI | *Ex vivo* perfused human lung | 5 or $10 \times 10^6$ human BM-MSCs, IB, 1–2 h post pneumonia | Restored alveolar fluid clearance<br>Decreased lung inflammation<br>Increased bacterial killing and reduced bacteremia<br>Increased alveolar macrophage phagocytosis | [32] |
| *P. aeruginosa* pneumonia | Sheep | 5 or $10 \times 10^6$ human BM-MSCs/kg, IV, 1 h post pneumonia | Improved $PaO_2/FiO_2$ ratio<br>Decreased lung water content | [33] |
| *E. coli* IP capsule | Mouse | $1 \times 10^6$ mouse AD-MSCs, retro-orbital injection, with capsule | Decreased splenocyte apoptosis<br>Decreased liver and pancreatic damage<br>Decreased proinflammatory cytokines<br>Increased IL-10 | [17] |
| *Host-barrier disruption* | | | | |
| CLP | Mouse | $1 \times 10^5$–$10^6$ human AD-MSCs, IP, 4 h post CLP | Improved survival<br>Decreased proinflammatory cytokines<br>Reduced inflammatory cell organ infiltration<br>Decreased bacterial burden in liver/spleen | [22] |
| CLP | Mouse | $1 \times 10^6$ mouse BM-MSCs, IV, 24 h before or 1 h post CLP | Reduced organ damage (dosed 24 h before CLP)<br>Decreased proinflammatory cytokines<br>Reprogrammed host macrophages to increase IL-10 production | [34] |

(*continued*)

**Table 55.1** (*Continued*)

| Model | Species | MSC methods | MSC effects | Reference |
|---|---|---|---|---|
| CLP | Mouse | $2 \times 10^5$ mouse BM-MSCs, IV, 6 h post CLP | Decreased mortality<br>Decreased proinflammatory cytokine levels<br>Prevented ALI and organ dysfunction<br>Improved bacterial clearance and enhanced phagocytosis of host immune cells | [27] |
| CLP | Mouse | $2.5 \times 10^5$ mouse BM-MSCs, IV, 6 h post CLP | Organ transcriptional responses of improved mitochondrial function, improved inflammatory profiles and preserved vascular integrity | [35] |
| CLP | Mouse | $5 \times 10^5$ 2 h post CLP and $2.5 \times 10^5$ 24 and 48 h post CLP Mouse compact bone MSCs, IV | Improved survival<br>Enhanced neutrophil phagocytosis<br>Increased bacterial clearance<br>Therapeutic effect dependent on neutrophils<br>Decreased organ injury in susceptible mice | [36] |
| CLP | Mouse | $1 \times 10^6$ mouse BM-MSCs IV, 3 h post CLP | Decreased acute kidney injury<br>Decreased renal inflammatory cytokines and neutrophil infiltration<br>Improved survival<br>Decreased blood bacterial load | [37] |
| CLP | Rat | $5 \times 10^6$ human BM or UC-MSCs, IV, 4 h post CLP | Increased regulatory T cells<br>Decreased proinflammatory cytokines | [38] |

ALI, acute lung injury; BAL, bronchoalveolar lavage; CM, conditioned media; IB, intrabronchial; IT, intratracheal; IP, intraperitoneal; IV: intravenous; TSS, toxic shock syndrome; UC, umbilical cord.

microbes, as well as in the production of inflammatory mediators during sepsis. They are also thought to contribute to the subsequent resolution of inflammation by releasing pro-resolving lipid mediators and clearing the site of injury of dead cells and debris to prevent further escalation of the inflammatory reaction [41]. Macrophages and monocytes also appear to be a major target in the protective effect of MSCs. In a Gram-negative model of peritoneal sepsis in mice, human MSCs reduced bacteremia in part by enhancing the phagocytic activity of blood monocytes [31].

MSCs have also been shown to alter cytokine production of macrophages. They were shown to induce IL-10-secreting macrophages in both *in vitro* and *in vivo* studies via $PGE_2$ signaling. In addition, they have been shown to decrease the production of the proinflammatory factors TNF-α, IL-1β, and IL-6 in macrophages by secreting soluble factors such as $PGE_2$ or TSG-6 [34,42]. The importance of the MSC and monocyte/macrophage interaction is highlighted by the fact that MSCs were no longer effective in CLP in mice lacking monocytes/macrophages [34].

It is well known that macrophages represent two different phenotypes: M1 and M2. The M1 or classically activated macrophages are considered to be proinflammatory macrophages with high expression of TNF-α, IL-1β, IL-6, and inducible NO synthase. In contrast, M2 or alternatively activated macrophages possess anti-inflammatory properties by releasing IL-10 and IL-4. Interestingly, several reports have indicated the ability of MSCs to change a macrophage phenotype from M1 to M2, which may explain the potential anti-inflammatory properties of MSCs [43–46]. Several other studies have further suggested that MSCs switch macrophages into a regulatory profile characterized by a low ability to produce inflammatory cytokines and a high ability to phagocytose apoptotic cells and bacteria [47]. Moreover, Dalli and Serhan demonstrated that M2 macrophages not only have anti-inflammatory actions but also pro-resolving properties by releasing pro-resolving lipid mediators such lipoxins, resolvins, protectins, and maresins [48]. However, the concept of MSCs effecting resolution of inflammation during infection needs further investigation.

The mechanisms by which MSCs regulate macrophages have not been full elucidated. It has been suggested that MSCs might act as regulators of the NLRP3 inflammasome. Inflammasomes are cytosolic multiprotein complexes that proteolytically activate caspase-1 in response to cellular "danger" signals [49]. Activated caspase-1 then processes proinflammatory cytokines, including IL-1β, into their biologically active and secreted forms. The best characterized inflammasome is the NLRP3 inflammasome. Human MSCs negatively regulated NLRP3 inflammasome activation in human and mouse macrophages that were exposed to LPS and adenosine-5′-triphosphate by decreasing mitochondrial ROS production that is critical for NLRP3 inflammasome activation [50].

Dendritic cells (DCs) are also a major part of the innate immune response. Upon maturation, DCs have an important role in antigen presentation to naive T lymphocytes. MSCs have been shown to affect DCs in several ways *in vitro*. MSCs can inhibit the maturation of DCs, impair the antigen-presenting function of mature DCs, and decrease the proinflammatory potential of DCs by inhibiting their production of

TNF-α [51]. Further *in vitro* effects of MSCs have been described. These include their ability to inhibit the proliferation and cytotoxicity of resting NK cells. These effects are largely mediated by $PGE_2$ and TGF-β [52]. However, it has recently been suggested that the tryptophan catabolizing enzyme IDO plays a role in the inhibition of NK cell proliferation. MSCs can express IDO that degrades essential tryptophan and results in accumulation of kynurenine breakdown products, resulting in reduced NK cell proliferation.

To summarize, it is clear that MSCs have profound and complex interactions with different types of innate immune system cells. The studies described here illustrate that MSCs play an important role in immune regulation during bacterial invasion.

## 55.4 Effects of mesenchymal stromal cells on the adaptive immune response

T lymphocytes are a major component of the adaptive immune system (see Chapter 34). Antigen-specific activation and differentiation of naive T cells results in the generation of a range of T cell phenotypes. Human BM-MSCs have been shown to suppress T cell proliferation *in vitro* [53]. This inhibition of T cell proliferation leads to a shift in T cells from a proinflammatory, interferon-γ-producing T cell to an anti-inflammatory, IL-4-producing state. MSCs have a similar effect on both memory and naive T cells, as well as on both $CD4^+$ T helper and $CD8^+$ cytotoxic T cell subsets. As with NK cells, TGF-β, IDO, and $PGE_2$ are thought to play a major role in inhibition of T cell proliferation by MSCs [54].

Another level at which MSCs modulate immune responses is through the induction of regulatory T cells. Regulatory T cells are a specialized population of T cells that suppress activation of the immune system and help to maintain a balance. MSCs have been reported to induce the production of IL-10 and to trigger the generation of regulatory T cells [55]. One study showed in rats subjected to CLP that the percentage of circulating regulatory T cells and the ratio of regulatory T cells/total T cells were elevated significantly in the group that received MSCs after CLP. In addition, it was demonstrated that the immunosuppressive capacity of regulatory T cells from rats with CLP-induced sepsis was diminished but could be restored by administration of MSCs [38].

The other major cell type involved in the adaptive immune response is the B cell, which produces antibodies. Most of the studies done to date indicate that MSCs inhibit B cell proliferation, chemotactic ability, and differentiation *in vitro*. In these experiments, B cell proliferation appeared to be inhibited by human MSCs through an arrest in the $G_0/G_1$ phase of the cell cycle and not through the induction of apoptosis [55,56].

The immunosuppressive properties of MSCs have been shown to be affected by specific disease-related tissue microenvironments [57]. These findings suggest that MSCs can modulate the intensity of the potentially harmful immune response without making the host susceptible to infections.

## 55.5 Antimicrobial activity of mesenchymal stromal cells

In addition to the bacterial clearance that occurs by enhanced monocyte and neutrophil phagocytosis, MSCs also exhibit benefit in models of sepsis via a direct antimicrobial activity. Several preclinical models have shown that MSC administration significantly reduced CLP-induced blood bacterial load [58]. In an *ex vivo* perfused human lung injured with live *E. coli* bacteria, alveolar fluid from lungs treated with MSCs had increased antimicrobial activity compared with lungs exposed to *E. coli* alone [32]. In addition, MSC CM was shown to possess direct antimicrobial activity in co-culture in *in vitro* experiments with *E. coli*, *P. aeruginosa*, and *Staphylococcus aureus*. These findings were confirmed in an *E. coli* pneumonia model in mice in which antimicrobial activity in the MSC CM was conserved, indicating the presence of a secreted soluble factor. The authors identified this factor as LL-37, a member of the cathelicidin peptide family [30].

## 55.6 Mesenchymal stromal cells and endothelial/epithelial dysfunction

Increasing evidence supports the substantial role of endothelial and/or epithelial cell dysfunction in the clinical deterioration that can be seen in sepsis. ANG-1 has been shown to enhance endothelial cell survival and barrier maintenance. Studies have shown that overexpression of ANG-1 in MSCs can lead to improvement in outcome after LPS-induced lung injury, including decreased inflammation and permeability [58]. In addition, not unlike leukocytes, MSCs express integrins and adhesion molecules and interact in a coordinated fashion with the vascular endothelium. These interactions allow MSCs to adhere and transmigrate into tissue at the site of injury [59]. While most MSC activity has been felt to be due to their secreted factors, MSCs have also been shown to differentiate to some degree after treatment with retinoic acid *in vitro* into epithelial cells [60]. This indicates that they may play a role in repair of the damaged epithelial barrier that can occur in sepsis.

## 55.7 Mesenchymal stromal cells and effect on organ injury in infection

Several interrelated mechanisms likely contribute to the pathogenesis of organ injury and dysfunction in sepsis. The early host response leads to the release of proinflammatory and procoagulant mediators. These mediators contribute to organ damage by priming immune cells, producing ROS and causing

circulatory derangement. The activation of these pathways also leads to endothelial and/or epithelial injury and dysfunction and microvasculature thrombosis [7]. In preclinical models, MSCs have shown benefit in organ injury caused by infection. These beneficial effects are now summarized by organ system.

The acute respiratory distress syndrome (ARDS) is a common cause of acute respiratory failure and is often caused by pneumonia and/or sepsis. Human MSCs or their CM were shown to restore lung protein permeability and reduce alveolar inflammation following *E. coli* endotoxin-induced lung injury in an *ex vivo* perfused human lung. This effect was mediated in part by the secretion of soluble factors, including KGF [12]. In addition, MSCs have been shown to reduce lung inflammation by inhibiting the transmigration of neutrophils into alveoli [26]. MSCs have also improved ALI in several other preclinical models of infection using live bacteria or following CLP in several species, including mice, rats, and sheep [28,33].

Islam *et al.* further investigated the molecular basis of the protective effect of MSCs in lung injury induced by LPS in mice. They demonstrated that mouse BM-MSCs infused into the trachea of mice attached to LPS-injured alveolar epithelial cells by forming connexin-43-containing gap junctions. They showed that, after attachment, labeled mitochondria were transferred from the MSCs to the LPS-injured alveolar cells through the formation of nanotubes and MVs. This was the first *in vivo* study to show that MSCs can repair tissue injury through the transfer of mitochondria [61,62] (and see Chapters 10, 20, 21, and 38).

In addition to the lung, MSCs have shown benefit on other organ injuries. Luo *et al.* showed that MSCs could ameliorate sepsis-associated acute kidney injury in mice undergoing CLP. The group of mice that received BM-MSCs intravenously after CLP had lower levels of kidney injury markers, improved tubular function, lower levels of proinflammatory cytokines and neutrophil infiltration in kidney tissue, and a higher survival rate than the control group [37].

Weil *et al.* showed that MSCs could attenuate decreased myocardial function and reduce myocardial inflammation in a model of endotoxemia in rats [27]. In a model of murine sepsis induced by IP placement of an *E. coli*-containing capsule, MSCs given intravenously after the infectious insult decreased splenic injury and splenic apoptosis. This study also demonstrated a reduction in liver damage as measured by serum alanine aminotransferase and in pancreatic damage as measured by serum amylase [17].

Németh *et al.* also showed that BM-MSCs reduced organ damage in the kidney, liver, and pancreas in a model of CLP. In this study, vascular permeability in the peritoneum, lung, liver, and kidney was also improved after CLP in the mice that were treated with MSCs [34].

Our laboratory has studied the effect of MSCs in a genetically modified mouse model that is susceptible to injury. These heme-oxygenase-1-deficient mice underwent CLP and were administered three doses of MSCs after the onset of polymicrobial sepsis. In these susceptible mice there was significantly less tissue injury seen on histological analysis of the bowel, kidney, liver, and spleen 24 h after CLP-induced sepsis in the group that received MSCs. The fact that MSCs could ameliorate CLP-associated organ injury in a mouse model that is more sensitive to organ injury further emphasizes the therapeutic impact they may have [36].

## 55.8 Mesenchymal stromal cell cytokine and growth factor production

The previous sections have described the ability of MSCs to alter the cytokine profile of resident cells such as macrophages. However, the potential beneficial activities of MSCs in sepsis may also be related to the MSC cytokine profile. MSCs are potent sources of the anti-inflammatory cytokines TGF-β, IL-10, and IL-13 [57]. The effects of MSCs on cytokine profiles have been seen in preclinical models of sepsis. MSC instillation decreased levels of the proinflammatory cytokine TNF-α and increased levels of the anti-inflammatory cytokines IL-10 and IL-13 in the BAL of mice undergoing endotoxin-induced lung injury [24]. In addition, injection of human AD-MSCs into mice that underwent CLP significantly ameliorated the clinical and histopathological severity of colitis, abrogating weight loss, diarrhea, and inflammation, and increasing survival. The therapeutic effect was associated with downregulation of type 1 T-helper-cell-driven inflammatory responses. AD-MSCs decreased a wide panel of inflammatory cytokines and chemokines and increased IL-10, acting on macrophages. The AD-MSCs also impaired type 1 T helper cell activation in both colonic mucosa and draining lymph nodes. The induction of IL-10-secreting regulatory T cells was partially involved in the therapeutic effect of the AD-MSCs. Moreover, AD-MSCs protected from severe sepsis by reducing the infiltration of inflammatory cells in various target organs and by downregulating the production of various inflammatory mediators [22].

The benefits of MSCs are further enhanced by MSC secretion of cytoprotective and proangiogenic factors such as VEGF, HGF, IGF-1, ANG-1, and FGF-2 [57]. Human MSCs have been shown to upregulate the production of these protective growth factors in response to noxious stimuli such as LPS. This process is largely mediated by MSC expression of TNF receptors [63].

The full extent of the neoangiogenic properties of MSCs continues to be investigated (see Chapters 25 and 26). Increased vasculature was found in MSC-treated wounds associated with elevated levels of VEGF-α and ANG-1. These data suggest that the high levels of proangiogenic growth factors and cytokines released by MSCs enhance neovascularization in the wound [64] (and see Chapters 58 and 59). In addition, the growth factors secreted by MSCs may be involved in mobilizing resident stem cell populations that exist in the adult heart, lung, liver, and kidney [65]. It is possible that MSCs can stimulate resident stem cell proliferation to aid in organ damage caused by sepsis.

## 55.9 Toll-like receptors and mesenchymal stromal cells

Inflammation in sepsis is largely initiated by TLRs that detect microbes, LPS shedding, and cellular contents released by injury to trigger an innate immune response. As a result, TLRs are an important part of the host response to infection. They are expressed by a variety of cells. It has been shown that their expression in human MSCs may play a role in mediating many of the beneficial actions of these cells [66]. This is further supported by the finding that the TLR3 ligand polyinosinic:polycytidylic acid could enhance the production of soluble factors in MSCs, including $PGE_2$ and IDO, and enhance the therapeutic effect of MSCs on CLP-induced sepsis [67]. Much remains to be learned about the role of TLRs in MSCs as there are contrasting and complex immune modulating effects reported thus far for TLR activation in MSCs. One group proposed a possible explanation for the different responses seen in MSCs after TLR signaling. They suggested that human MSCs, like monocytes, are polarized by downstream TLR signaling into two homogenously acting phenotypes that they classified as MSC1 (proinflammatory) and MSC2 (immunosuppressive) [68].

## 55.10 Mesenchymal stromal cell homing

MSCs have the ability to home to sites of tissue injury and inflammation when given via IV or IP routes [44]. For example, one study found that in response to CLP-induced polymicrobial sepsis, the greatest concentrations of MSCs were in the lung, kidney, and spleen [34]. This is a quality that makes them appealing for the treatment of infection and sepsis, where tissue injury and inflammation are widespread. While the mechanisms by which MSCs are recruited to tissues and cross the endothelial layer are not yet fully understood, chemokines and their receptors are involved. SDF-1 (also known as C—X—C motif chemokine ligand 12) and its corresponding receptor CXCR4 appear to be the most significant mediators of this process in MSCs [69]. In addition, many of the molecules known to be involved in the tethering, rolling, adhesion, and transmigration of leukocytes from the bloodstream into tissues are known to be expressed on MSCs. These include integrins, selectins, and chemokine receptors [54] (and see Chapters 22 and 23).

## 55.11 Mesenchymal stromal cell response to oxidative stress

ROS and reactive nitrogen species (RNS) are produced in the setting of infection and act as antimicrobial agents that can directly destroy microbial pathogens. However, during sepsis, excess production of ROS and RNS can be detrimental. It is not uncommon for the oxidative stress burden to persist after eradication of the organism. One study demonstrated that the *in vitro* treatment of MSCs with LPS induced production of ROS and RNS that ultimately resulted in redox stress. These events triggered in MSCs a cascade of responses driving adaptive remodeling and resistance to oxidative stress. (Oxidative stress reflects an imbalance between the systemic manifestation of ROS and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Further, some reactive oxidative species act as cellular messengers in redox signaling. Thus, oxidative stress can cause disruptions in normal mechanisms of cellular signaling.) The data described here suggested that MSCs are able to activate a prosurvival pathway under conditions of oxidative stress that may add to their ability to modulate septic conditions [70].

## 55.12 Paracrine effects of mesenchymal stromal cells

As described in this chapter, the beneficial effects of MSCs derive predominantly from their capacity to secrete paracrine factors that modulate immune responses and alter the responses of cells to injury (see also Chapter 37). However, it is poorly understood whether the cell itself is necessary for this therapeutic response or if the secreted factors alone would exert the same benefit. Studies suggest that the distribution of MSCs might be essential for the beneficial effect. As mentioned earlier, MSCs can home to damaged organs to protect them from injury. However, intravenously infused MSCs initially accumulate in the lungs, trapped in the first capillary bed that they encounter after IV infusion, probably due to their large size. This suggests that MSCs exert their protective effects via a paracrine effect. In one study, human MSCs were infused intravenously into mice and most of the cells were trapped in the lung. However, after myocardial infarction these MSCs decreased inflammatory responses in the heart and improved cardiac function by secreting the anti-inflammatory protein TSG-6 [71]. The paracrine effect of MSCs is further supported by the fact that MSC CM has shown benefit in some preclinical models of sepsis [37].

MSCs release circular membrane fragments called exosomes or MVs that are biologically active because of the presence in them of mRNA or microRNA [72]. Zhu *et al.* showed that MVs released from BM-MSCs improved several indices of endotoxin-induced ALI in mice. KGF appeared to be partially responsible for the MV effect [28]. There has been significant work done on MVs and exosomes in models of hypoxic pulmonary hypertension that may relate to the ARDS that can occur after sepsis. Lee *et al* showed that exosomes mediate the cytoprotective action of MSCs in these models [73]. This work suggests that MVs may be therapeutically effective. However, the cell itself may have further benefits, as it is able to respond to the complex and changing conditions of sepsis.

## 55.13 Transcriptomic analysis of mesenchymal stromal cell therapy in sepsis

Despite the benefits that MSCs have shown in experimental models of sepsis, the cellular and molecular mechanisms mediating their beneficial effects are complex. A recent network analysis of transcriptional responses of target organs in a CLP model of sepsis provided some insight into these mechanisms. In this study mice underwent CLP and 6 h later were treated with MSCs. Total RNA from the lungs, hearts, kidneys, livers, and spleens was analyzed for common transcriptional responses. These responses identified three common effects of MSC administration: attenuation of sepsis-induced mitochondrial-related functional derangement, downregulation of TLR innate immune proinflammatory responses, and preservation of endothelial/vascular integrity. This study illustrated that the protective effect of MSC therapy in sepsis likely involves a wide range of complementary activities [35].

## 55.14 Summary

Research utilizing preclinical models of sepsis has provided a greater understanding of the beneficial properties of MSCs for this serious disorder. MSCs show promise in the treatment of bacterial infections and sepsis by their ability to interact with the innate and adaptive immune responses, alter cytokine profiles, enhance bacterial clearance, home to injured tissue, limit inflammation and organ injury, decrease cell apoptosis, and improve endothelial/epithelial dysfunction [7]. While MSCs have been trialed in a variety of human disease conditions, clinical trials are just beginning in human sepsis and infection. There is currently a clinical trial of mesenchymal cells in patients with septic shock and severe neutropenia (https://clinicaltrials.gov/ct2/show/NCT01849237). In addition, as lung injury is a known complication of infection and sepsis, much information can be gained from the use of MSCs for ARDS. A recent phase I dose-escalation, multicenter trial was conducted to study the effects of human allogeneic BM-MSCs for the treatment of ARDS. The authors found that a single IV infusion of these cells was well tolerated. A phase II trial is ongoing [74]. While more mechanistic information is necessary to further understand how to translate these findings into clinical use, MSCs continue to show significant promise as a therapy for bacterial infections and sepsis.

## References


1 Vincent, J.L. and E. Abraham. The last 100 years of sepsis. *American Journal of Respiratory and Critical Care Medicine*, 2006;**173**(3):256–263.

2 Annane, D., E. Bellissant, and J.M. Cavaillon. Septic shock. *Lancet*, 2005;**365**(9453):63–78.

3 Pinsky, M.R. Sepsis: a pro- and anti-inflammatory disequilibrium syndrome. *Contributions to Nephrology*, 2001;**132**:354–366.

4 Abraham, E. and M. Singer. Mechanisms of sepsis-induced organ dysfunction. *Critical Care Medicine*, 2007;**35**(10):2408–2416.

5 Baron, R.M., M.J. Baron, and M.A. Perrella. Pathobiology of sepsis: are we still asking the same questions? *American Journal of Respiratory Cell and Molecular Biology*, 2006;**34**(2):129–134.

6 Riedemann, N.C., R.F. Guo, and P.A. Ward. Novel strategies for the treatment of sepsis. *Nature Medicine*, 2003;**9**(5):517–524.

7 Weil, B.R., T.A. Markel, J.L. Herrmann, *et al.* Stem cells in sepsis. *Annals of Surgery*, 2009;**250**(1):19–27.

8 Kusadasi, N. and A.B. Groeneveld. A perspective on mesenchymal stromal cell transplantation in the treatment of sepsis. *Shock*, 2013;**40**(5):352–357.

9 Prockop, D.J. Repair of tissues by adult stem/progenitor cells (MSCs): controversies, myths, and changing paradigms. *Molecular Therapy*, 2009;**17**(6):939–946.

10 Buras, J.A., B. Holzmann, and M. Sitkovsky. Animal models of sepsis: setting the stage. *Nature Reviews. Drug Discovery*, 2005;**4**(10):854–865.

11 Schumann, R.R., S.R. Leong, G.W. Flaggs, *et al.* Structure and function of lipopolysaccharide binding protein. *Science*, 1990;**249**(4975):1429–1431.

12 Lee, J.W., X. Fang, N. Gupta, *et al.* Allogeneic human mesenchymal stem cells for treatment of *E. coli* endotoxin-induced acute lung injury in the *ex vivo* perfused human lung. *Proceedings of the National Academy of Sciences of the United States of America*, 2009;**106**(38):16357–16362.

13 Chen, L.Y., W.W. Pan, M. Chen, *et al.* Synergistic induction of inflammation by bacterial products lipopolysaccharide and fMLP: an important microbial pathogenic mechanism. *Journal of Immunology*, 2009;**182**(4):2518–2524.

14 Deitch, E.A. Animal models of sepsis and shock: a review and lessons learned. *Shock*, 1998;**9**(1):1–11.

15 Nemzek, J.A., K.M. Hugunin, and M.R. Opp. Modeling sepsis in the laboratory: merging sound science with animal well-being. *Comparative Medicine*, 2008;**58**(2):120–128.

16 Toky, V., S. Sharma, B.B. Arora, and S. Chhibber. Establishment of a sepsis model following implantation of *Klebsiella pneumoniae*-infected fibrin clot into the peritoneal cavity of mice. *Folia Microbiologica*, 2003;**48**(5):665–669.

17 Pedrazza, L., A. Lunardelli, C. Luft, *et al.* Mesenchymal stem cells decrease splenocytes apoptosis in a sepsis experimental model. *Inflammation Research*, 2014;**63**(9):719–728.

18 Rittirsch, D., M.S. Huber-Lang, M.A. Flierl, and P.A. Ward. Immunodesign of experimental sepsis by cecal ligation and puncture. *Nature Protocols*, 2009;**4**(1):31–36.

19 Doi, K., A. Leelahavanichkul, P.S. Yuen, and R.A. Star. Animal models of sepsis and sepsis-induced kidney injury. *The Journal of Clinical Investigation*, 2009;**119**(10):2868–2878.

20 Muenzer, J.T., C.G. Davis, B.S. Dunne, *et al.* Pneumonia after cecal ligation and puncture: a clinically relevant "two-hit" model of sepsis. *Shock*, 2006;**26**(6):565–570.

21 Zhu, H., Z.K. Guo, X.X. Jiang, *et al.* A protocol for isolation and culture of mesenchymal stem cells from mouse compact bone. *Nature Protocols*, 2010;**5**(3):550–560.

22 Gonzalez-Rey, E., P. Anderson, M.A. Gonzalez, *et al.* Human adult stem cells derived from adipose tissue protect against experimental colitis and sepsis. *Gut*, 2009;**58**(7):929–939.

23 Wannemuehler, T.J., M.C. Manukyan, B.D. Brewster, *et al.* Advances in mesenchymal stem cell research in sepsis. *The Journal of Surgical Research*, 2012;**173**(1):113–126.

24 Gupta, N., X. Su, B. Popov, *et al.* Intrapulmonary delivery of bone marrow-derived mesenchymal stem cells improves survival and attenuates endotoxin-induced acute lung injury in mice. *Journal of Immunology*, 2007;**179**(3):1855–1863.

25 Mei, S.H., J.J. Haitsma, C.C. Dos Santos, *et al.* Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *American Journal of Respiratory and Critical Care Medicine*, 2010;**182**(8):1047–1057.

26 Xu, J., C.R. Woods, A.L. Mora, *et al.* Prevention of endotoxin-induced systemic response by bone marrow-derived mesenchymal stem cells in mice. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 2007;**293**(1):L131–L141.

27 Weil, B.R., M.C. Manukyan, J.L. Herrmann, *et al.* Mesenchymal stem cells attenuate myocardial functional depression and reduce systemic and myocardial inflammation during endotoxemia. *Surgery*, 2010;**148**(2):444–452.

28 Zhu, Y.G., X.M. Feng, J. Abbott, *et al.* Human mesenchymal stem cell microvesicles for treatment of *Escherichia coli* endotoxin-induced acute lung injury in mice. *Stem Cells*, 2014;**32**(1):116–125.

29 Kim, H., I. Darwish, M.F. Monroy, *et al.* Mesenchymal stromal (stem) cells suppress pro-inflammatory cytokine production but fail to improve survival in experimental staphylococcal toxic shock syndrome. *BMC Immunology*, 2014;**15**:1.

30 Krasnodembskaya, A., Y. Song, X. Fang, *et al.* Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells*, 2010;**28**(12):2229–2238.

31 Krasnodembskaya, A., G. Samarani, Y. Song, *et al.* Human mesenchymal stem cells reduce mortality and bacteremia in Gram-negative sepsis in mice in part by enhancing the phagocytic activity of blood monocytes. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, 2012;**302**(10):L1003–L1013.

32 Lee, J.W., A. Krasnodembskaya, D.H. McKenna, *et al.* Therapeutic effects of human mesenchymal stem cells in *ex vivo* human lungs injured with live bacteria. *American Journal of Respiratory and Critical Care Medicine*, 2013;**187**(7):751–760.

33 Asmussen, S., H. Ito, D.L. Traber, *et al.* Human mesenchymal stem cells reduce the severity of acute lung injury in a sheep model of bacterial pneumonia. *Thorax*, 2014;**69**(9):819–825.

34 Németh, K., A. Leelahavanichkul, P.S. Yuen, *et al.* Bone marrow stromal cells attenuate sepsis via prostaglandin $E_2$-dependent reprogramming of host macrophages to increase their interleukin-10 production. *Nature Medicine*, 2009;**15**(1):42–49.

35 Dos Santos, C.C., S. Murthy, P. Hu, *et al.* Network analysis of transcriptional responses induced by mesenchymal stem cell treatment of experimental sepsis. *The American Journal of Pathology*, 2012;**181**(5):1681–1692.

36 Hall, S.R., K. Tsoyi, B. Ith, *et al.* Mesenchymal stromal cells improve survival during sepsis in the absence of heme oxygenase-1: the importance of neutrophils. *Stem Cells*, 2013;**31**(2):397–407.

37 Luo, C.J., F.J. Zhang, L. Zhang, *et al.* Mesenchymal stem cells ameliorate sepsis-associated acute kidney injury in mice. *Shock*, 2014;**41**(2):123–129.

38 Chao, Y.H., H.P. Wu, K.H. Wu, *et al.* An increase in $CD3^+CD4^+CD25^+$ regulatory T cells after administration of umbilical cord-derived mesenchymal stem cells during sepsis. *PLoS ONE*, 2014;**9**(10):e110338.

39 Brandau, S., M. Jakob, K. Bruderek, *et al.* Mesenchymal stem cells augment the anti-bacterial activity of neutrophil granulocytes. *PLoS ONE*, 2014;**9**(9):e106903.

40 Raffaghello, L., G. Bianchi, M. Bertolotto, *et al.* Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. *Stem Cells*, 2008;**26**(1):151–162.

41 Serhan, C.N. Pro-resolving lipid mediators are leads for resolution physiology. *Nature*, 2014;**510**(7503):92–101.

42 Choi, H., R.H. Lee, N. Bazhanov, *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood*, 2011;**118**(2):330–338.

43 Abumaree, M.H., M.A. Al Jumah, B. Kalionis, *et al.* Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages. *Stem Cell Reviews*, 2013;**9**(5):620–641.

44 Melief, S.M., E. Schrama, M.H. Brugman, *et al.* Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of monocytes toward anti-inflammatory macrophages. *Stem Cells*, 2013;**31**(9):1980–1891.

45 Cho, D.I., M.R. Kim, H.Y. Jeong, *et al.* Mesenchymal stem cells reciprocally regulate the M1/M2 balance in mouse bone marrow-derived macrophages. *Experimental & Molecular Medicine*, 2014;**46**:e70.

46 Gao, S., F. Mao, B. Zhang, *et al.* Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-κB and signal transducer and activator of transcription 3 pathways. *Experimental Biology and Medicine*, 2014;**239**(3):366–375.

47 Maggini, J., G. Mirkin, I. Bognanni, *et al.* Mouse bone marrow-derived mesenchymal stromal cells turn activated macrophages into a regulatory-like profile. *PLoS ONE*, 2010;**5**(2):e9252.

48 Dalli, J. and C.N. Serhan, Specific lipid mediator signatures of human phagocytes: microparticles stimulate macrophage efferocytosis and pro-resolving mediators. *Blood*, 2012;**120**(15):e60–e72.

49 Gross, O., C.J. Thomas, G. Guarda, and J. Tschopp The inflammasome: an integrated view. *Immunological Reviews*, 2011;**243**(1):136–151.

50 Oh, J.Y., J.H. Ko, H.J. Lee, *et al.* Mesenchymal stem/stromal cells inhibit the NLRP3 inflammasome by decreasing mitochondrial reactive oxygen species. *Stem Cells*, 2014;**32**(6):1553–1563.

51 Jiang, X.X., Y. Zhang, B. Liu, *et al.* Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood*, 2005;**105**(10):4120–4126.

52 Uccelli, A., L. Moretta, and V. Pistoia, Mesenchymal stem cells in health and disease. *Nature Reviews. Immunology*, 2008;**8**(9):726–736.

53 Di Nicola, M., C. Carlo-Stella, M. Magni, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood*, 2002;**99**(10):3838–3843.

54 Chamberlain, G., J. Fox, B. Ashton, and J. Middleton. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells*, 2007;**25**(11):2739–2749.

55 Aggarwal, S. and M.F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood*, 2005;**105**(4): 1815–1822.

56 Corcione, A., F. Benvenuto, E. Ferretti, *et al.* Human mesenchymal stem cells modulate B-cell functions. *Blood*, 2006;**107**(1): 367–372.

57 Ma, S., N. Xie, W. Li, *et al.* Immunobiology of mesenchymal stem cells. *Cell Death and Differentiation*, 2014;**21**(2):216–225.

58 Mei, S.H., S.D. McCarter, Y. Deng, *et al.* Prevention of LPS-induced acute lung injury in mice by mesenchymal stem cells overexpressing angiopoietin 1. *PLoS Medicine*, 2007;**4**(9):e269.

59 Semon, J.A., L.H. Nagy, C.B. Llamas, *et al.* Integrin expression and integrin-mediated adhesion *in vitro* of human multipotent stromal cells (MSCs) to endothelial cells from various blood vessels. *Cell and Tissue Research*, 2010;**341**(1):147–158.

60 Ricciardi, M., G. Malpeli, F. Bifari, *et al.* Comparison of epithelial differentiation and immune regulatory properties of mesenchymal stromal cells derived from human lung and bone marrow. *PLoS ONE*, 2012;**7**(5):e35639.

61 Prockop, D.J. Mitochondria to the rescue. *Nature Medicine*, 2012;**18** (5):653–654.

62 Islam, M.N., S.R. Das, M.T. Emin, *et al.* Mitochondrial transfer from bone-marrow-derived stromal cells to pulmonary alveoli protects against acute lung injury. *Nature Medicine*, 2012;**18**(5):759–765.

63 Nauta, A.J. and W.E. Fibbe. Immunomodulatory properties of mesenchymal stromal cells. *Blood*, 2007;**110**(10):3499–3506.

64 Chen, L., E.E. Tredget, P.Y. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE*, 2008;**3**(4):e1886.

65 Hatzistergos, K.E., H. Quevedo, B.N. Oskouei, *et al.* Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circulation Research*, 2010;**107**(7):913–922.

66 Pevsner-Fischer, M., V. Morad, M. Cohen-Sfady, *et al.* Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood*, 2007;**109**(4):1422–1432.

67 Zhao, X., D. Liu, W. Gong, *et al.* The toll-like receptor 3 ligand, poly (I:C), improves immunosuppressive function and therapeutic effect of mesenchymal stem cells on sepsis via inhibiting MiR-143. *Stem Cells*, 2014;**32**(2):521–533.

68 Waterman, R.S., S.L. Tomchuck, S.L. Henkle, and A.M. Betancourt. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE*, 2010;**5**(4):e10088.

69 Wynn, R.F., C.A. Hart, C. Corradi-Perini, *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood*, 2004;**104**(9):2643–2645.

70 Gorbunov, N.V., B.R. Garrison, D.P. McDaniel, *et al.* Adaptive redox response of mesenchymal stromal cells to stimulation with lipopolysaccharide inflammagen: mechanisms of remodeling of tissue barriers in sepsis. *Oxidative Medicine and Cellular Longevity*, 2013; 2013: 186795.

71 Lee, R.H., A.A. Pulin, M.J. Seo, *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell*, 2009;**5**(1):54–63.

72 Sdrimas, K. and S. Kourembanas. MSC microvesicles for the treatment of lung disease: a new paradigm for cell-free therapy. *Antioxidants & Redox Signaling*, 2014;**21**(13):1905–1915.

73 71 Lee, C., S.A. Mitsialis, M. Aslam, *et al.* Exosomes mediate the cytoprotective action of mesenchymal stromal cells on hypoxia-induced pulmonary hypertension. *Circulation*, 2012;**126**(22):2601–2611.

74 Wilson, J.G., K.D. Liu, H. Zhuo, *et al.* Mesenchymal stem (stromal) cells for treatment of ARDS: a phase 1 clinical trial. *The Lancet. Respiratory Medicine*, 2015;**3**(1):24–32.

CHAPTER 56

# The use of mesenchymal stromal cells in solid organ transplantation

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## 56.1 Introduction

Organ transplantation is the only definitive treatment for many critical diseases of the liver, kidney, heart, pancreas, and lungs. Although it is the primary therapeutic option at present, transplanted patients have to deal with the numerous side effects of life-long dependence on immunosuppressive drugs, and these drugs still fail to prevent chronic rejection of the transplanted organ in many cases. The risk of developing cancer and opportunistic infections is also markedly increased in solid organ transplant (SOT) recipients receiving long-term immunosuppressive therapy. Cancer and opportunistic infections cannot be completely avoided since they result from the immunosuppressive drugs used posttransplant that affect not only the anti-graft response but also the entire immune response. Finding a way to establish donor-specific immunological tolerance without the need for nonspecific immunosuppression remains one of the major goals in transplantation medicine [1,2]. Another important aim is the improvement of graft survival and function. Overall, graft survival is about 15 years, but the increasing shortage of organs has led to the use of expanded criteria for donor organs often donated by older individuals, which are less robust organs than those donated by younger donors.

Mesenchymal stromal cells (MSCs) are currently being evaluated in SOT with the hope of achieving more selective immunosuppression, better graft function, and longer graft survival.

## 56.2 Potential effects of mesenchymal stromal cells in solid organ transplantation

Potential MSC benefits to SOT could be mediated through three main mechanisms: immunomodulation, tissue repair, and antioxidative effects. Moreover, MSCs are able to selectively home to the sites of tissue injury and inflammation, including the intestine, kidney, lung, liver, thymus, and skin [3]. Mechanisms underlying this homing include interactions between soluble mediators such as stromal cell-derived factor-1, hyaluronic acid, macrophage inflammatory protein-1(α), hepatocyte growth

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factor (HGF) and their respective MSC receptors, C–X–C chemokine receptor type 4 (CXCR4), CD44, C–C chemokine receptor 1, and c-Met [4] (and see Chapters 22 and 23).

## 56.3 Immunomodulation

The immunosuppressive properties of MSCs have been widely studied and include inhibition of T cell proliferation and function, inhibition of dendritic cell (DC) maturation, and induction of regulatory T cells (Tregs), DCs, and macrophages. For example, Bartholomew *et al.* demonstrated the ability of MSCs to inhibit lymphocyte proliferation *in vitro* and to prolong skin graft survival *in vivo* [5]. The importance of immunomodulation by MSCs in SOT is illustrated by the fact that donor-derived, autologous, and unrelated third-party MSCs seem to have different effects in transplantation models [6]. Transplantation of human leukocyte antigen (HLA)-nonidentical MSCs is enabled by their low immunogenicity. However, while some authors performed mismatched allogeneic MSC transplantation with no signs of alloreactivity against donor MSCs [7,8], others observed induction of memory T cell responses and immune rejection of MSCs in major histocompatibility complex mismatched hosts [9–11]. Pretransplant infusion of donor-derived MSCs may lead to sensitization to donor antigens and hence to accelerated graft rejection, or, on the contrary, may desensitize the host to donor antigens. Dosing and timing of MSC infusion may play a role in this immune balancing act [12]. So far, some data suggest that MSCs sharing major histocompatibility complex antigens with the donor graft may be superior for inducing long-term tolerance [6]. The effects of MSCs on the innate immune system and the adaptive immune system are described in detail in Chapters 33 and 34 respectively.

## 56.4 Tissue and organ regeneration

The regenerative effects of MSCs were initially attributed to their multilineage differentiation capacity, such as in liver disease in which MSC administration enabled liver regeneration in animal models of terminal liver failure or metabolic diseases [13,14]. This could be useful in partial liver transplantation (LT) (split graft or living donor LT), a technique that is being increasingly used because of the shortage of donor organs. MSCs also demonstrated multiple beneficial effects in repairing acute kidney injury in animal models of acute renal failure through differentiation toward endothelial or smooth muscle cell lineages [15] or through a reduction of apoptosis and an increased proliferation of renal cells [16].

More recently it has been demonstrated that the beneficial effects of MSCs in these settings are mediated by their ability to secrete trophic factors that induce proliferation and differentiation of progenitor cells at the site of injury, rather than from transdifferentiation [6]. For example, allogeneic human MSCs are able to restore alveolar fluid clearance in human lungs through the secretion of keratinocyte growth factor [17]. The importance of these soluble factors secreted by MSCs is also illustrated by the fact that MSC-conditioned medium (MSC-CM) has been found effective in reducing inflammation and improving survival in an animal model of LT [18]. Further information on the MSC secretome is given in Chapters 18 and 19.

## 56.5 Prevention of ischemia–reperfusion injury

The term ischemia–reperfusion injury (IRI) defines inflammatory lesions observed when tissues are reperfused after an ischemic period, as happens after declamping in transplant surgery. Mechanisms involve endothelium dysfunction with increased permeability, production of oxygen radicals and inflammatory mediators, and expression of adhesion molecules. These phenomena lead to a stimulation of both innate and adaptive immune responses that are responsible for organ damage [19].

Because of their ability to migrate into hypoxic or inflamed tissues and to downregulate excessive immune responses, the potential beneficial effects of MSCs on IRI have been investigated in animal models of kidney [20–23], liver [24,25], and lung [26] IRI with promising results. For example, it has been demonstrated that hyaluronic acid accumulating in the kidney following ischemic injury stimulates the migration of MSCs through interaction with the surface receptor CD44 [27]. Complex paracrine interactions seem involved in this protective effect, with downregulation of proinflammatory signals such as tumor necrosis factor-α, transforming growth factor-β, interleukin (IL)-1β, and IL-6 and upregulation of anti-inflammatory cytokines such as IL-10 [24], resulting in MSC-mediated suppression of oxidative stress and inhibition of apoptosis [25].

## 56.6 Mesenchymal stromal cell administration in solid organ transplantation

### 56.6.1 Kidney transplantation

Kidney transplantation is the best option for patients suffering from end-stage kidney diseases, improving survival and quality of life compared with maintenance dialysis [28,29]. The site of placement of a transplanted kidney is shown in Figure 56.1.

However, long-term immunosuppressive treatments are necessary to prevent graft rejection. Induction therapy generally includes an IL-2 receptor antagonist such as basiliximab or a lymphocyte-depleting agent such as rabbit antithymocyte globulin (ATG) or muromonab-CD3 (OKT-3). A combination of immunosuppressive medications is used as maintenance therapy, including calcineurin inhibitors (CNIs) such as tacrolimus

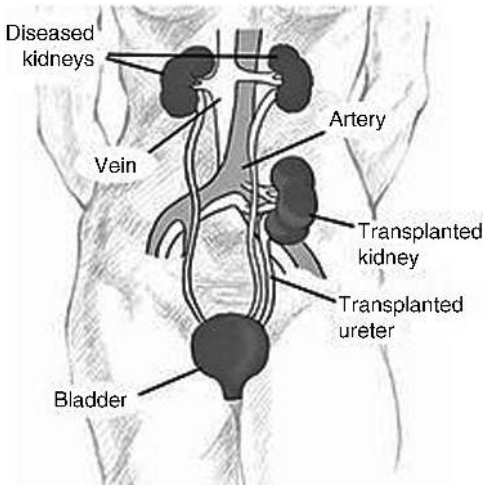



**Figure 56.1** Kidney location after transplantation. *Source:* nih.gov.

or cyclosporine (CSP), antiproliferative agents such as mycophenolate or azathioprine, corticosteroids (prednisolone) and/or mammalian target of rapamycin inhibitors such as sirolimus or everolimus. The main causes of posttransplant morbidity and mortality are cardiovascular diseases, infections, malignancies, and graft dysfunction.

#### 56.6.1.1 Animal studies

MSCs have demonstrated their ability to downregulate the immune response after kidney transplantation in several animal studies. Zhang *et al* transplanted Lewis rats with Wistar rat kidney and bone marrow (BM)-MSCs. Intravenous $1 \times 10^7$ BM-MSCs were given 1 week before transplantation, immediately after reperfusion, and 1 and 2 weeks posttransplant and compared with the effects of MSCs alone, CSP alone, and a combination of MSCs and CSP. They found that MSCs were able to preserve graft function, as assessed by serum creatinine level, and to prolong animal survival, but were not as effective as CSP [30].

Monotherapy with third-party BM-MSCs infused directly into the renal artery of the transplanted kidney ($3 \times 10^6$ cells injected soon after reperfusion) was studied in a syngeneic and allogeneic model of rat kidney transplantation. Compared with placebo, MSC-treated rats had better kidney function as assessed by serum creatinine level, creatinine clearance rate, proteinuria and diuresis, reduced histological damage as assessed by the degree of tubulitis and vasculitis, and a reduced inflammatory infiltrate with fewer $ED1^+$ and $CD8^+$ cells [31]. ED1 is a rat marker of activated monocytes, macrophages, DCs, and microglial cells and is also known as CD68. Tubulitis is a defining feature for the diagnosis and management of acute renal allograft rejection.

The efficacy of syngeneic MSCs to prevent IRI was also demonstrated in a rat kidney transplantation model with prolonged cold ischemia. Rats received CSP 1.5 mg/kg/per day for 2 days and three intravenous injections of $2.5 \times 10^6$ or $5.0 \times 10^6$ MSCs 7 days before, immediately after, and 1 day after transplantation. MSC administration resulted in reduced intragraft gene expression of proinflammatory cytokines, chemokines, and intercellular adhesion molecule-1, and in a reduced infiltration of antigen-presenting cells in the renal allograft [32].

The mechanisms of MSC-induced allograft tolerance are not yet fully elucidated, but generation of Tregs and tolerogenic DCs and production of indoleamine 2,3-dioxygenase appear to be involved in mice [33]. The importance of the route of administration has been pointed out in rats, as intra-arterial injection was shown to be more efficient than intravenous injection, with a more rapid functional recovery as assessed by serum creatinine levels and reduced inflammatory infiltrate in the graft [34]. These findings suggested that a high concentration of MSCs at the site of immunological injury may be crucial for the efficacy of MSCs in this setting.

With regard to the timing of MSC administration, experiments in mice have shown that syngeneic MSCs injected intravenously after transplantation 2 days posttransplant localized mainly in the graft, where they were responsible for local inflammatory reaction and failed to enhance graft survival, while MSCs injected 7 days and 1 day before transplantation localized in lymphoid organs, promoting Treg expansion and significantly prolonging allograft survival [35]. However, it has been shown in rats that a delayed third-party MSC injection was also beneficial, protecting the kidney allograft from injury development as shown by reduced interstitial fibrosis and tubular atrophy, lower inflammatory cell infiltration, and improved balance between proinflammatory and anti-inflammatory cytokines [36].

The long-term safety of MSC administration has been questioned by one group, who observed an unexpected increase in MSC-related life-threatening events in rats, including thrombotic microangiopathy, infarctions, and infections [37]. So far there has been no other report of such MSC-related adverse events in transplant recipients.

#### 56.6.1.2 Human studies

The first studies on human cells showed that *in vitro* donor-derived adipose MSCs were able to significantly inhibit recipient antidonor reactivity and proliferation of $CD4^+$ and $CD8^+$ T lymphocyte subsets in mixed lymphocyte reactions [38]. Combined MSC and hematopoietic stem cell co-transplantation compared with hematopoietic stem cell transplantation alone produced significantly higher peripheral blood chimerism, better kidney transplant function as assessed by serum creatinine levels, and improved survival [39]. Intra-osseous injection of donor MSCs into the BM at a dose of $1 \times 10^6$ cells/kg at the time of kidney transplantation was evaluated in a pilot study of seven patients undergoing living donor kidney transplantation. The procedure was safe, but three patients had biopsy-proven acute rejection that was controlled with corticosteroid pulse therapy. Donor-specific T cell proliferation was observed in two patients, while Treg priming responses were observed in two others associated with increased IL-10 levels in the peripheral blood [40].

#### 56.6.1.3 Mesenchymal stromal cells versus conventional induction therapy

Perico *et al.* evaluated the pretransplant infusion of autologous BM-MSCs in two living-related kidney transplant recipients. One patient had corticosteroid-sensitive acute cellular rejection of the kidney 2 weeks posttransplant, but both patients had excellent renal function at 1 year follow-up. Circulating memory $CD8^+$ T cell numbers and donor-specific $CD8^+$ T cell cytolytic responses were reduced. Compared with patients receiving basiliximab induction therapy, $CD4^+$ $FoxP3^+$ Treg expansion was not increased [41].

Tan *et al.* reported a randomized controlled trial of living donor kidney transplantation in 156 patients who were divided into three groups: a control group receiving antibody induction therapy versus two groups receiving autologous BM-MSCs at a dose of $1–2 \times 10^6$/kg administered at the time of kidney graft reperfusion and 2 weeks later plus either low-dose or standard-dose CNI treatment. Graft survival was similar, but MSC infusion resulted in fewer acute rejections at 6 months, no corticosteroid-resistant rejection, accelerated renal function recovery, and a reduced rate of opportunistic infections. However, acute rejection rates and glomerular filtration rates were not different at 1 year postransplant [42].

#### 56.6.1.4 Mesenchymal stromal cells versus conventional maintenance therapy

Perico *et al.* also investigated the effect of autologous BM-MSCs in two living-related kidney transplant recipients 7 days after transplantation. Increasing levels of $CD4^+$ $CD25^{high}$ $FoxP3^+$ $CD127^+$ Tregs and inhibition of memory/effector $CD8^+$ T cell expansion were observed, resulting in a long-term tolerogenic environment compared with the control group. However, patients suffered from engraftment syndrome with transient renal dysfunction a few days after MSC administration, and biopsies showed a focal inflammatory infiltrate with neutrophils and MSCs and complement C3 deposition [43]. (Engraftment syndrome characterized by fever, rash, pulmonary edema, weight gain, liver and renal dysfunction, and/or encephalopathy usually occurs at the time of neutrophil recovery after hematopoietic cell transplantation).

Another recent pilot study with 12 patients receiving living-donor related kidneys compared donor-derived BM-MSC infusions with a first dose of $5 \times 10^6$ cells into the renal allograft artery at the time of kidney transplantation and a second intravenous dose of $2 \times 10^6$ cells/kg 1 month later plus low-dose (50% of usual) tacrolimus versus a control group who received no MSCs and standard doses of tacrolimus. Patients also received mycophenolate mofetil (MMF) and methylprednisolone. All patients had stable renal function at 1 year posttransplant despite reduced tacrolimus doses in the MSC group, and only one episode of acute rejection occurred (in the control group). Significantly increased B cell levels were observed in the MSC group 3 months after transplantation. There were no MSC-related adverse events [44].

#### 56.6.1.5 Mesenchymal stromal cells as treatment for kidney rejection

In a phase I trial, Reinders *et al.* studied two intravenous infusions of $10^6$ autologous BM-MSCs per kilogram as treatment for kidney rejection in six patients. MSC infusions were well tolerated, and no treatment-related serious adverse events were reported. Biopsies were performed after MSC administration in two patients and showed resolution of tubulitis without interstitial fibrosis/tubular atrophy. Five patients had downregulation of peripheral blood mononuclear cell proliferation and three patients developed an opportunistic viral infection [45].

#### 56.6.1.6 Mesenchymal stromal cells as a desensitizing treatment

In a report of three cases, infusion of $50 \times 10^6$ donor-derived MSCs led to successful desensitization of patients with a positive lymphocyte cross-match before kidney transplantation [46].

#### 56.6.1.7 Ongoing trials

##### *56.6.1.7.1 Mesenchymal stromal cells in addition to conventional therapy*

The Liège trial (NCT01429038) is a single-center, open-label prospective phase I–II study that includes 10 kidney transplant recipients receiving a kidney from a deceased donor (and 10 liver transplant recipients who will be described later). In addition to induction therapy of standard immunosuppression with anti-IL-2 antibodies and maintenance therapy of tacrolimus, MMF, and corticosteroids, patients receive an intravenous infusion of third-party MSCs at a dose of $1.5–3.0 \times 10^6$/kg on postoperative day 3 ($\pm$2 days). The 10 study patients will be compared at 1 year posttransplant with a control group of 10 similar kidney transplant recipients. The primary endpoint is safety of the MSC infusion, including infusional toxicity and incidence of infections and cancers. Secondary endpoints are patient and graft survival; graft function assessed by the number of posttransplant hemodialysis episodes required and serum creatinine levels; biopsy-proven rejection rates using the Banff classification; feasibility and safety of weaning or decreasing immunosuppression, recipient immune function as assessed by T cell blood populations, including Tregs, T-cell receptor excision circle quantification, Vβ repertoire diversity, pathogen-specific T cells, anti-organ donor HLA antibodies; and potential development of anti-MSC donor HLA antibodies.

The first Bergamo trial (NCT00752479) is studying the effects of syngeneic *ex vivo* expanded MSCs in HLA-mismatched (one or two haplotype mismatches) living donor kidney transplantation. Patients are randomized into two groups. One group receives an intravenous injection of $2 \times 10^6$ MSCs/kg at the time of kidney transplantation. All patients receive conventional induction treatment with basiliximab, rabbit ATG and methylprednisolone and maintenance treatment with CSP and MMF. Patients will be followed for 12 months. Primary outcomes include percentage inhibition of memory and/or naive T cell responses, induction of donor-reactive T cell anergy, and levels of

circulating Tregs. Secondary outcomes are safety of the MSC infusion, graft function, and graft rejection.

The second Bergamo trial (NCT02012153) is investigating the effect of autologous *ex vivo* expanded BM-MSCs in HLA-mismatched (one or two haplotype mismatches) living donor kidney transplantation. MSCs are administered intravenously on the day before kidney transplantation at a dose of $2 \times 10^6$ MSCs/kg. Patients are followed for trial parameters for 1 year. Outcomes evaluated at 6 and 12 months include levels of circulating naive cells, memory cells, and Tregs, T cell function in mixed lymphocyte reaction, urinary forkhead box P3 (FOXP3) mRNA expression, and adverse events.

#### *56.6.1.7.2 Mesenchymal stromal cells versus conventional induction therapy*

The Fuzhou study (NCT00658073) is evaluating autologous MSCs as an alternative to antibody induction therapy in renal transplantation. Patients are randomized into three groups, two of them receiving two intravenous injections of MSCs immediately after releasing the renal artery clamp and 2 weeks after transplantation, while the third group receives standard induction therapy with anti-IL-2 receptor antibody. All patients will receive maintenance therapy with CNIs, MMF, and corticosteroids, but one of the MSC groups receives a reduced dose (80% less) of CNIs. Patients will be followed for trial parameters for 1 year. Primary outcomes are incidence of acute rejection and early renal function recovery. Secondary outcomes are patient and graft survival, and prevalence of adverse events.

#### *56.6.1.7.3 Mesenchymal stromal cells versus conventional maintenance therapy*

In a Leiden trial (NCT02057965), intravenous infusions of autologous BM-MSCs are being evaluated as maintenance therapy in renal transplantation with a kidney from a deceased, a living unrelated, or a HLA-nonidentical living-related donor older than 50 years of age. Patients are randomized into two groups. One group will receive MSCs and everolimus versus tacrolimus and everolimus. Patients in the MSC group will receive two injections of $1–2 \times 10^6$ MSCs/kilogram at 6 and 7 weeks after transplantation. After the second MSC infusion, tacrolimus is tapered off and withdrawn over a 2-week period. The primary outcome is kidney fibrosis at 6 months compared with 4 weeks posttransplant. Secondary outcomes evaluated at 6 months posttransplant are renal function and proteinuria, complications (including opportunistic infections and cardiovascular disease), adverse effects, a composite endpoint of efficacy failure (biopsy-proven acute rejection, graft loss, or death, and immunological parameters such as donor-specific antibodies.

#### *56.6.1.7.4 Mesenchymal stromal cells as treatment for kidney rejection*

Another Leiden trial (NCT00734396) is studying the safety and efficacy of autologous BM-MSCs to treat renal transplant rejection in HLA-DR mismatched kidney graft recipients. Patients with subclinical rejection and/or an increase in interstitial fibrosis/tubular atrophy in the renal biopsy at 4 weeks or 6 months receive two intravenous injections of $1–2 \times 10^6$ MSCs/kg 7 days apart. Primary outcomes are safety and feasibility of MSC administration at 2 years, while secondary outcomes are efficacy parameters, including the presence of late acute rejection in the 6-month biopsy compared with the 4-week biopsy, Sirius red staining for renal cortical matrix accumulation, and immunologic response before and after MSC infusion.

### 56.6.2 Liver transplantation

Since the pioneering experience in the 1960s LT has become established as the standard treatment of many end-stage liver diseases, including cirrhosis, primary liver cancer, fulminant hepatic failure, and a number of other metabolic or congenital hepatic diseases. Expected patient survival after LT is now more than 80% at 1 year and 60–70% at 5 years, dependent on the different indications for transplantation [47]. The excellent results of LT have led to a shortage of liver grafts that has been partially fulfilled by the development of living-related LT, and the procurement of liver grafts from brain-dead donors, aged cadaveric donors, or donors procured after cardiac death [48]. A human liver photographed at autopsy is shown in Figure 56.2.

Immunosuppressive regimens include CNIs, antimetabolites, mammalian target of rapamycin inhibitors, corticosteroids, and antibody-based therapies. A combination of CNIs and antimetabolites with tapering doses of corticosteroids during the first month is generally used to prevent graft rejection, while treatment of rejection usually comprises boluses of intravenous corticosteroids or ATG for corticosteroid-resistant rejection.

#### 56.6.2.1 Animal studies

Syngeneic BM-MSCs have shown their ability to home to the liver and to alleviate acute immunologic rejection in an orthotopic (normal location) rat LT model [49]. Similarly,

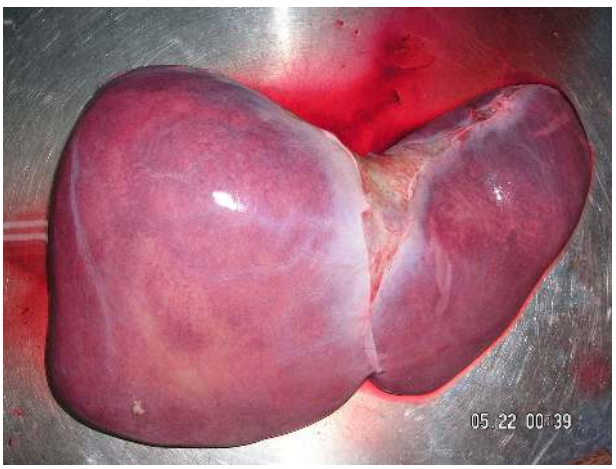



**Figure 56.2** Human liver. *Source:* http://commons.wikimedia.org/wiki/File:Human_Hepar.jpg#/media/File:Human_Hepar.jpg.

adipose-tissue-derived MSCs infused intravenously at day −7 and day +3 as well as during the operation via the portal vein were able to significantly decrease acute rejection as assessed by reduced serum IL-2 levels and increased serum IL-10 levels as well as reduced hepatocyte apoptosis [50]. MSCs also demonstrated their efficacy in a liver xenotransplantation model (orthotopic guinea pig to rat LT), enabling increased survival and alleviating acute rejection compared with placebo or with the powerful corticosteroid dexamethasone [51].

MSCs from different donors have shown equivalent effects in a rat LT model. Recipient-, donor-, or third-party-derived MSCs were infused intravenously at the time of surgery and once daily for 3 days thereafter. MSC administration resulted in significantly improved recipient survival, inhibition of allograft rejection based on histological analysis and induction of $CD4^+$ $CD25^+$ $Foxp3^+$ Tregs, with no significant difference between the three MSC-treated groups [52].

Du *et al.* studied the efficacy of the intravenous injection of MSC-CM in a 50% reduced-size rat LT model. In the MSC-CM group the authors observed a significantly lower release of liver injury biomarkers, a reduction in hepatocyte apoptosis, an increased proliferation of hepatocytes and sinusoidal endothelial cells, and reduced signs of inflammation in the graft as assessed by lower expression of several proinflammatory cytokines, reduced neutrophil infiltration and Kupffer cell activation and increased expression of vascular endothelial growth factor and matrix metallopeptidase (or metalloproteinase) 9. These observations were associated with increased survival in the MSC-CM group compared with the control group [18]. (Kuppfer cells were first observed by Karl Wilhelm von Kupffer in 1876 and are hepatic macrophages.)

In an attempt to improve MSC homing, the same group used MSCs overexpressing CXCR4 in a 50% reduced size rat LT model. MSCs reduced the release of liver injury biomarkers and apoptosis of hepatocytes. Although unable to further reduce liver injury, CXCR4 overexpression successfully enhanced MSC homing and hepatocyte proliferation, resulting in a significant survival benefit [53].

MSCs were also efficient in preventing, but not treating, acute graft-versus-host disease (GVHD) following LT. Rats that received donor- or recipient-derived BM-MSCs from day 0 to day 6 after LT ($5 \times 10^6$ MSCs/day) had a significantly longer survival and no typical LT-associated GVHD symptoms, while administration of MSCs after the onset of symptoms was ineffective for treating GVHD. The efficacy of MSC therapy was associated with higher Treg ratios in the peripheral blood [54].

Another use of MSCs in LT has been the *in vivo* delivery of engineered growth factors or cytokines. Intraportal infusion of $5 \times 10^6$ syngeneic HGF-expressing MSCs immediately after liver reperfusion was performed in a 30% small-for-size rat LT model. HGF-expressing MSCs prevented liver failure and improved liver function and liver weight recovery. Moreover, HGF enhanced the incorporation of MSCs into injured livers, and the engrafted cells and their progeny produced albumin [55]. In another study the same group showed that HGF-expressing MSCs significantly inhibited the formation of liver fibrosis in rats undergoing small-for-size rat LT with synergistic effects of the MSCs and HGF [56].

Similarly, genetic delivery of IL-10 by IL-10-engineered-MSCs has been tested. Compared with placebo, intravenous injection of syngeneic IL-10-transduced MSCs 30 min after transplantation resulted in longer survival, lower Banff scores, increased expression of the Treg-associated cytokines IL-10 and TGF-β1, and the transcription factor FOXP3, as well as decreased expression of T helper (Th)17 T-cell-related cytokines IL-17, IL-6, interferon-γ, tumor necrosis factor-α, and IL-23 and the retinoic-acid-related orphan receptor gamma (RORγ) transcription factor (RORγt) [57]. RORγ is a protein that in humans is encoded by the *RORC* (RAR-related orphan receptor C) gene. RORγ is member of the nuclear receptor family of transcription factors. It is essential for lymphoid organogenesis, in particular lymph nodes and Peyer's patches, but not the spleen. RORγ also plays an important regulatory role in thymopoiesis, by reducing apoptosis of thymocytes and promoting thymocyte differentiation into proinflammatory Th17 cells. It also plays a role in inhibiting apoptosis of undifferentiated T cells and promoting their differentiation into Th17 cells, possibly by downregulating the expression of Fas ligand and IL2 respectively. RORγt drives production of the cytokine granulocyte-macrophage colony-stimulating factor in Th cells, which is essential for the effector phase of autoimmune neuroinflammation.

#### 56.6.2.2 Ongoing trials in humans

The Liège trial (NCT01429038), mentioned earlier, is a single-center, open-label prospective phase I–II study that includes 10 liver transplant recipients (as well as the 10 kidney transplant recipients described earlier). In addition to standard immunosuppression with tacrolimus, MMF, and corticosteroids, patients receive an intravenous infusion of third-party MSCs at a dose of $1.5–3.0 \times 10^6$ MSCs/kg on the third postoperative day (±2 days). The 10 study patients will be followed for trial parameters for 1 year and compared with a control group of 10 similar liver recipients. The primary endpoints are safety of the MSC infusion and the incidence of infections and cancers. The secondary endpoints are patient and graft survival; graft function as assessed by serum bilirubin levels, prothrombin time, hepatic transaminase, and GGT levels; biopsy-proven (Banff classification) rejection rates; feasibility and safety of weaning or decreasing immunosuppression; recipient immune function as assessed by T cell blood populations, including Tregs, T-cell receptor excision circle quantification, Vβ repertoire diversity, pathogen-specific T cells, and anti-organ donor HLA antibodies; and potential development of anti-MSC donor HLA antibodies.

The Beijing trial (NCT01690247) and the Guangdong trial (NCT02223897) are studying the addition of intravenous injections of $1 \times 10^6$ umbilical cord MSCs/kg in addition to conventional therapy. MSCs are administered once every fourth week

for 12 weeks in the first trial and once per week for the first month followed by once per month for 6 months in the second trial. In the Beijing trial, patients are followed for trial parameters for 48 weeks. Outcomes are rates of acute rejection and early liver function recovery, patient and graft survival, and incidence of adverse events. The Guangdong trial is focused on ischemic-type biliary lesions, and outcomes after 18 months include the incidence of ischemic-type biliary lesions, and changes in hepatic enzymes and biliary blood supply as assessed by contrast-enhanced ultrasound examination.

### 56.6.3 Heart transplantation

Since the first human heart transplantation in 1967 this treatment has been the best option for selected patients with end-stage heart failure not remediable by more conservative measures. Despite significant improvements in survival since the introduction of current immunosuppression and better prevention and treatment of infections, there remain major complications, including allograft vasculopathy and other classical complications of solid organ transplantation, including graft failure, graft rejection, opportunistic infections, and malignancies.

#### 56.6.3.1 Animal studies

In rats, mesenchymal progenitor cells have demonstrated their ability to migrate into heart allografts after systemic administration [58] and to participate in tissue repair during chronic rejection [59]. The effects of MSC administration on heart allograft survival are still unknown. Some authors have shown that in rats MSCs did not prolong allograft survival [60] and even accelerated allograft rejection when used alone [61] or when added to low-dose CSP [62], while other studies demonstrated increased heart allograft survival using MSCs alone [63–66] or when MSCs were added to MMF [60,67]. A combination of MSCs and low-dose sirolimus has enabled long-term heart graft survival of more than 100 days with normal histology [65]. The mechanisms of MSC effects on heart allografts are not yet elucidated, but seem to include $CD4^+$ $CD25^+$ $Foxp3^+$ Treg and tolerogenic DC expansion [64,65], interferon-γ-induced suppression of T cell proliferation [60,61], and inhibition of endothelial activation with intercellular adhesion molecule 1 and E-selectin downregulation [61].

No trials in humans have been conducted yet.

### 56.6.4 Lung transplantation

Single or bilateral lung transplantation is a viable treatment option for patients with various end-stage lung diseases, such as chronic obstructive pulmonary disease, idiopathic pulmonary fibrosis, cystic fibrosis, emphysema due to alpha-1 antitrypsin deficiency, and idiopathic pulmonary arterial hypertension. Lung transplant recipients are exposed to complications that include primary graft dysfunction, infections, malignancies, and both acute and chronic rejection. Chronic lung allograft manifests as a bronchiolitis obliterans syndrome (BOS). Endothelin-1-stimulated MSCs may play a pathologic role in this phenomenon by differentiating locally into myofibroblastic cells [68]. However, another group demonstrated that resident lung MSCs isolated from human lung allografts inhibited T cell proliferation *in vitro* [69].

There is only one preclinical study of the administration of MSCs in lung transplantation, and this demonstrated the ability of IL-10-engineered MSCs to prevent lung IRI in mice [26].

The immunomodulatory properties of MSCs are currently being assessed in patients with BOS after lung transplantation. In the Prince Charles Hospital trial in Brisbane, Australia (NCT01175655), patients with BOS will receive MSCs from a third-party donor at a dose of $2 \times 10^6$/kg twice weekly for 2 weeks. Safety, pulmonary function, and survival will be evaluated at 12 months. In a Mayo Clinic trial (NCT02181712), patients with BOS will receive an intravenous injection of $2–4 \times 10^6$ allogeneic MSCs/kg. Adverse events and pulmonary function will be assessed after 2 weeks.

### 56.6.5 Pancreas and islet transplantation

Pancreas or islet transplantation is performed in order to restore glucose-regulated endogenous insulin secretion, which allows insulin independence and improves glycemic control in patients with diabetes mellitus. However, pancreas or islets available for transplantation are scarce, and long-term pancreatic transplant survival rates are unsatisfactory, partly due to the lack of markers for pancreas graft rejection. As in other organ transplantations, long-term immunosuppression with several drugs is often required posttransplant.

MSCs have the ability to differentiate into insulin-producing cells both *in vitro* [70] and *in vivo* in animals [71,72] and humans [73,74]. Moreover, autologous [75], syngeneic [76–78] or allogeneic [78–80] MSCs have demonstrated immunomodulatory properties in animal models of islet transplantation. Complex mechanisms are involved including secretion of soluble factors (such as matrix metalloproteinase-2 and -9 [76] and IL-10 [81]), improvement of graft revascularization [77], and effects on immune cells (including the inhibition of maturation and function of DCs [82], downregulation of memory T cells [80], and induction of regulatory T cells [83]). Moreover, in non-human primates, co-transplantation of allogeneic donor MSCs and islet cells led to significantly enhanced islet engraftment and function at 1 month posttransplant, and additional infusions of donor or third-party MSCs successfully treated rejection [84]. In addition to these effects, MSCs provide a supportive microenvironmental niche through the secretion of paracrine factors and the deposition of extracellular matrix *in vitro* [85,86], in pretransplantation co-cultures [87], and *in vivo* [88]. Finally, as in LT, MSCs are able to prevent IRI in animal models of islet transplantation [89,90].

There is only one ongoing clinical trial on co-transplantation of allogeneic islets and autologous MSCs. Primary outcomes are exogenous insulin requirement, glycated hemoglobin, and glucose and C-peptide levels. Secondary outcomes are liver and

kidney function, portal vein ultrasound appearance, autoantibodies, and complete blood count (NCT00646724).

### 56.6.6 Bowel transplantation

There is one case report of successful administration of third-party-derived BM-MSCs to treat severe refractory bowel dysfunction secondary to infection after bowel transplantation [91]. More recently, Doğan *et al.* gave an intra-arterial infusion of autologous BM-MSCs (three doses of $1 \times 10^6$ MSCs/kg) at the time of reperfusion and on days 15 and 30 posttransplant in six patients undergoing small intestine transplantation. All patients received anti-thymocyte immunoglobulin and corticosteroids as induction therapy and a CNI using low-dose tacrolimus as maintenance therapy. Four patients developed acute rejection. Three did not survive, of whom one did not respond to bolus corticosteroid therapy and two developed severe fungal and bacterial infections after corticosteroids. Two patients did not experience rejection and had no complications with good quality of life during the 30 months of trial follow-up [92].

## 56.7 Conclusions

Studies are providing increasing evidence of MSC benefits in solid organ transplantation. These benefits appear to be obtained by a combination of three major properties of MSCs: immunomodulation, tissue/organ repair, and prevention of IRI. Complex mechanisms are involved, and further research is required to elucidate them. Convincing animal studies in kidney transplantation and LT, and to a lesser extent in lung and islet transplantation, have led to the initiation of human trials. Initial results are promising in kidney transplantation. In heart transplantation, conflicting data have been obtained and more research is needed in animal models before human clinical trials can be initiated. Major goals in this field are reduction of graft rejection rates, improvement of organ and patient survival, and avoidance of treatment-related adverse effects through dose reduction or withdrawal of immunosuppressive drugs.

## References


1 N. Pilat, C. Klaus, E. Schwaiger, T. Wekerle. Hurdles to the induction of tolerogenic mixed chimerism. *Transplantation* 2009;**87**:S79–S84.

2 F. C. Popp, E. Eggenhofer, P. Renner, *et al.* Mesenchymal stem cells can affect solid organ allograft survival. *Transplantation* 2009;**87**:S57–S62.

3 S. M. Devine, C. Cobbs, M. Jennings, *et al.* Mesenchymal stem cells distribute to a wide range of tissues following systemic infusion into nonhuman primates. *Blood* 2003;**101**:2999–3001.

4 L. A. Marquez-Curtis, A. Janowska-Wieczorek. Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis. *Biomed Res Int* 2013;**2013**:561098.

5 A. Bartholomew, C. Sturgeon, M. Siatskas, *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;**30**:42–48.

6 M. J. Hoogduijn, F. C. Popp, A. Grohnert, *et al.* Advancement of mesenchymal stem cell therapy in solid organ transplantation (MISOT). *Transplantation* 2010;**90**:124–126.

7 K. Le Blanc, C. Götherström, O. Ringdén, *et al.* Fetal mesenchymal stem-cell engraftment in bone after *in utero* transplantation in a patient with severe osteogenesis imperfecta. *Transplantation* 2005;**79**:1607–1614.

8 M. Sundin, O. Ringdén, B. Sundberg, *et al.* No alloantibodies against mesenchymal stromal cells, but presence of anti-fetal calf serum antibodies, after transplantation in allogeneic hematopoietic stem cell recipients. *Haematologica* 2007;**92**:1208–1215.

9 A. J. Nauta, G. Westerhuis, A. B. Kruisselbrink, *et al.* Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a non-myeloablative setting. *Blood* 2006;**108**:2114–2120.

10 K. H. Grinnemo, A. Månsson, G. Dellgren, *et al.* Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *J Thorac Cardiovasc Surg* 2004;**127**:1293–1300.

11 N. Eliopoulos, J. Stagg, L. Lejeune, *et al.* Allogeneic marrow stromal cells are immune rejected by MHC class I- and class II-mismatched recipient mice. *Blood* 2005;**106**:4057–4065.

12 M. Crop, C. Baan, W. Weimar, M. Hoogduijn. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. *Transpl Int* 2009;**22**:365–376.

13 F. C. Popp, P. Piso, H. J. Schlitt, M. H. Dahlke. Therapeutic potential of bone marrow stem cells for liver diseases. *Curr Stem Cell Res Ther* 2006;**1**:411–418.

14 T. K. Kuo, S. P. Hung, C. H. Chuang, *et al.* Stem cell therapy for liver disease: parameters governing the success of using bone marrow mesenchymal stem cells. *Gastroenterology* 2008;**134**:2111–2121.

15 J. Chen, H. C. Park, F. Addabbo, *et al.* Kidney-derived mesenchymal stem cells contribute to vasculogenesis, angiogenesis and endothelial repair. *Kidney Int* 2008;**74**:879–889.

16 M. Morigi, M. Introna, B. Imberti, *et al.* Human bone marrow mesenchymal stem cells accelerate recovery of acute renal injury and prolong survival in mice. *Stem Cells* 2008;**26**:2075–2082.

17 D. F. McAuley, G. F. Curley, U. I. Hamid, *et al.* Clinical grade allogeneic human mesenchymal stem cells restore alveolar fluid clearance in human lungs rejected for transplantation. *Am J Physiol Lung Cell Mol Physiol* 2014;**306**:L809–L815.

18 Z. Du, C. Wei, K. Cheng, *et al.* Mesenchymal stem cell-conditioned medium reduces liver injury and enhances regeneration in reduced-size rat liver transplantation. *J Surg Res* 2013;**183**:907–915

19 P. Erpicum, J. M. Krzesinski, F. Jouret. Place de l'AMP-activated protein kinase dans le preconditionnement ischemique renal. *Nephrol Ther* 2013;**10**:17–24.

20 P. Semedo, P. M. Wang, T. H. Andreucci, *et al.* Mesenchymal stem cells ameliorate tissue damages triggered by renal ischemia and reperfusion injury. *Transplant Proc* 2007;**39**:421–423.

21 F. Tögel, Z. Hu, K. Weiss, *et al.* Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *Am J Physiol Renal Physiol* 2005;**289**:F31–F42.

22 F. Tögel, K. Weiss, Y. Yang, *et al.* Vasculotropic, paracrine actions of infused mesenchymal stem cells are important to the recovery from acute kidney injury. *Am J Physiol Renal Physiol* 2007;**292**:F1626–F1635.

23 P. Erpicum, O. Detry, L. Weekers, *et al.* Mesenchymal stromal cell therapy in conditions of renal ischaemia/reperfusion. *Nephrol Dial Transplant* 2014;**29**:1487–1493.

24 C. K. Sun, C. L. Chang, Y. C. Lin, *et al.* Systemic administration of autologous adipose-derived mesenchymal stem cells alleviates hepatic ischemia–reperfusion injury in rats. *Crit Care Med* 2012;**40**:1279–1290.

25 G. Jin, G. Qiu, D. Wu, *et al.* Allogeneic bone marrow-derived mesenchymal stem cells attenuate hepatic ischemia–reperfusion injury by suppressing oxidative stress and inhibiting apoptosis in rats. *Int J Mol Med* 2013;**31**:1395–1401.

26 E. Manning, S. Pham, S. Li, *et al.* Interleukin-10 delivery via mesenchymal stem cells: a novel gene therapy approach to prevent lung ischemia-reperfusion injury. *Hum Gene Ther* 2010;**21**:713–727.

27 M. B. Herrera, B. Bussolati, S. Bruno, *et al.* Exogenous mesenchymal stem cells localize to the kidney by means of CD44 following acute tubular injury. *Kidney Int* 2007;**72**:430–441.

28 P. Schnuelle, D. Lorenz, M. Trede, F. J. Van Der Woude. Impact of renal cadaveric transplantation on survival in end-stage renal failure: evidence for reduced mortality risk compared with hemodialysis during long-term follow-up. *J Am Soc Nephrol* 1998;**9**:2135–2141.

29 F. K. Port, R. A. Wolfe, E. A. Mauger, *et al.* Comparison of survival probabilities for dialysis patients vs cadaveric renal transplant recipients. *JAMA* 1993;**270**:1339–1343.

30 W. Zhang, C. Qin, Z. M. Zhou. Mesenchymal stem cells modulate immune responses combined with cyclosporine in a rat renal transplantation model. *Transplant Proc* 2007;**39**:3404–3408.

31 M. De Martino, S. Zonta, T. Rampino, *et al.* Mesenchymal stem cells infusion prevents acute cellular rejection in rat kidney transplantation. *Transplant Proc* 2010;**42**:1331–1335.

32 Y. Hara, M. Stolk, J. Ringe, *et al. In vivo* effect of bone marrow-derived mesenchymal stem cells in a rat kidney transplantation model with prolonged cold ischemia. *Transpl Int* 2011;**24**:1112–1123.

33 W. Ge, J. Jiang, J. Arp, *et al.* Regulatory T-cell generation and kidney allograft tolerance induced by mesenchymal stem cells associated with indoleamine 2,3-dioxygenase expression. *Transplantation* 2010;**90**:1312–1320.

34 S. Zonta, M. De Martino, G. Bedino, *et al.* Which is the most suitable and effective route of administration for mesenchymal stem cell-based immunomodulation therapy in experimental kidney transplantation: endovenous or arterial? *Transplant Proc* 2010;**42**:1336–1340.

35 F. Casiraghi, N. Azzollini, M. Todeschini, *et al.* Localization of mesenchymal stromal cells dictates their immune or proinflammatory effects in kidney transplantation. *Am J Transplant* 2012;**12**: 2373–2383.

36 M. Franquesa, E. Herrero, J. Torras, *et al.* Mesenchymal stem cell therapy prevents interstitial fibrosis and tubular atrophy in a rat kidney allograft model. *Stem Cells Dev* 2012;**21**:3125–3135.

37 M. Koch, A. Lehnhardt, X. Hu, *et al.* Isogeneic MSC application in a rat model of acute renal allograft rejection modulates immune response but does not prolong allograft survival. *Transpl Immunol* 2013;**29**:43–50.

38 M. J. Crop, C. C. Baan, S. S. Korevaar, *et al.* Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation* 2009;**87**:896–906.

39 A. V. Vanikar, H. L. Trivedi, A. Feroze, *et al.* Effect of co-transplantation of mesenchymal stem cells and hematopoietic stem cells as compared to hematopoietic stem cell transplantation alone in renal transplantation to achieve donor hypo-responsiveness. *Int Urol Nephrol* 2011;**43**:225–232.

40 H. Lee, J. B. Park, S. Lee, *et al.* Intra-osseous injection of donor mesenchymal stem cell (MSC) into the bone marrow in living donor kidney transplantation; a pilot study. *J Transl Med* 2013; **11**:96.

41 N. Perico, F. Casiraghi, E. Gotti, *et al.* Mesenchymal stromal cells and kidney transplantation: pretransplant infusion protects from graft dysfunction while fostering immunoregulation. *Transpl Int* 2013;**26**:867–878.

42 J. Tan, W. Wu, X. Xu, *et al.* Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. *JAMA* 2012;**307**:1169–1177.

43 N. Perico, F. Casiraghi, M. Introna, *et al.* Autologous mesenchymal stromal cells and kidney transplantation: a pilot study of safety and clinical feasibility. *Clin J Am Soc Nephrol* 2011;**6**:412–422.

44 Y. Peng, M. Ke, L. Xu, *et al.* Donor-derived mesenchymal stem cells combined with low-dose tacrolimus prevent acute rejection after renal transplantation: a clinical pilot study. *Transplantation* 2013;**95**:161–168.

45 M. E. Reinders, J. W. de Fijter, H. Roelofs, *et al.* Autologous bone marrow-derived mesenchymal stromal cells for the treatment of allograft rejection after renal transplantation: results of a phase I study. *Stem Cells Transl Med* 2013;**2**:107–111.

46 G. Saadi, F. Fadel, M. El Ansary, S. A. El-Hamid. Mesenchymal stem cell transfusion for desensitization of positive lymphocyte cross-match before kidney transplantation: outcome of 3 cases. *Cell Prolif* 2013;**46**:121–126.

47 R. Adam, E. Hoti. Liver transplantation: the current situation. *Semin Liver Dis* 2009;**29**:3–18.

48 O. Detry, V. Donckier, V. Lucidi, *et al.* Liver transplantation from donation after cardiac death donors: initial Belgian experience 2003–2007. *Transpl Int* 2010;**24**:611–618.

49 Z. F. Hong, X. J. Huang, Z. Y. Yin, *et al.* Immunosuppressive function of bone marrow mesenchymal stem cells on acute rejection of liver allografts in rats. *Transplant Proc* 2009;**41**:403–409.

50 C. D. Wan, R. Cheng, H. B. Wang, T. Liu. Immunomodulatory effects of mesenchymal stem cells derived from adipose tissues in a rat orthotopic liver transplantation model. *Hepatobiliary Pancreat Dis Int* 2008;**7**:29–33.

51 J. W. Wang, Y. B. Liu, B. Xu, *et al.* The study on immunomodulation of donor mesenchymal stem cells on discordant liver xenotransplantation. *Zhonghua Wai Ke Za Zhi* 2005;**43**:1254–1258 (in Chinese).

52 Y. Wang, A. Zhang, Z. Ye, *et al.* Bone marrow-derived mesenchymal stem cells inhibit acute rejection of rat liver allografts in association with regulatory T-cell expansion. *Transplant Proc* 2009;**41**: 4352–4356.

53 Z. Du, C. Wei, J. Yan, *et al.* Mesenchymal stem cells overexpressing C–X–C chemokine receptor type 4 improve early liver regeneration of small-for-size liver grafts. *Liver Transpl* 2013;**19**:215–225.

54 X. Xia, W. Chen, T. Ma, *et al.* Mesenchymal stem cells administered after liver transplantation prevent acute graft-versus-host disease in rats. *Liver Transpl* 2012;**18**:696–706.

55 Y. Yu, A. H. Yao, N. Chen, *et al.* Mesenchymal stem cells over-expressing hepatocyte growth factor improve small-for-size liver grafts regeneration. *Mol Ther* 2007;**15**:1382–1389.

56 Y. Yu, L. Lu, X. Qian, *et al.* Antifibrotic effect of hepatocyte growth factor-expressing mesenchymal stem cells in small-for-size liver transplant rats. *Stem Cells Dev* 2010;**19**:903–914.

57 J. Niu, W. Yue, Y. Song, *et al.* Prevention of acute liver allograft rejection by IL-10-engineered mesenchymal stem cells. *Clin Exp Immunol* 2014;**176**:473–484.

58 G. D. Wu, J. A. Nolta, Y. S. Jin, *et al.* Migration of mesenchymal stem cells to heart allografts during chronic rejection. *Transplantation* 2003;**75**:679–685.

59 G. D. Wu, M. E. Bowdish, Y. S. Jin, *et al.* Contribution of mesenchymal progenitor cells to tissue repair in rat cardiac allografts undergoing chronic rejection. *J Heart Lung Transplant* 2005;**24**: 2160–2169.

60 E. Eggenhofer, P. Renner, Y. Soeder, *et al.* Features of synergism between mesenchymal stem cells and immunosuppressive drugs in a murine heart transplantation model. *Transpl Immunol* 2011;**25**: 141–147.

61 E. Eggenhofer, J. F. Steinmann, P. Renner, *et al.* Mesenchymal stem cells together with mycophenolate mofetil inhibit antigen presenting cell and T cell infiltration into allogeneic heart grafts. *Transpl Immunol* 2011;**24**:157–163.

62 S. Inoue, F. C. Popp, G. E. Koehl, *et al.* Immunomodulatory effects of mesenchymal stem cells in a rat organ transplant model. *Transplantation* 2006;**81**:1589–1595.

63 H. P. Zhou, D. H. Yi, S. Q. Yu, *et al.* Administration of donor-derived mesenchymal stem cells can prolong the survival of rat cardiac allograft. *Transplant Proc* 2006;**38**:3046–3051.

64 F. Casiraghi, N. Azzollini, P. Cassis, *et al.* Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. *J Immunol* 2008;**181**:3933–3946.

65 W. Ge, J. Jiang, M. L. Baroja, *et al.* Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *Am J Transplant* 2009;**9**:1760–1772.

66 S. M. Wu, W. X. Zhang, M. H. Wang, *et al.* Proteomic analysis of the immunosuppressive effects of mesenchymal stem cells in a rat heart transplantation model. *Adv Clin Exp Med* 2013;**22**:785–794.

67 F. C. Popp, E. Eggenhofer, P. Renner, *et al.* Mesenchymal stem cells can induce long-term acceptance of solid organ allografts in synergy with low-dose mycophenolate. *Transpl Immunol* 2008;**20**:55–60.

68 M. Salama, P. Jaksch, O. Andrukhova, *et al.* Endothelin-1 is a useful biomarker for early detection of bronchiolitis obliterans in lung transplant recipients. *J Thorac Cardiovasc Surg* 2010;**140**:1422–1427.

69 L. Jarvinen, L. Badri, S. Wettlaufer, *et al.* Lung resident mesenchymal stem cells isolated from human lung allografts inhibit T cell proliferation via a soluble mediator. *J Immunol* 2008;**181**:4389–4396.

70 Y. Sun, L. Chen, X. G. Hou, *et al.* Differentiation of bone marrow-derived mesenchymal stem cells from diabetic patients into insulin-producing cells *in vitro*. *Chin Med J (Engl)* 2007;**120**:771–776.

71 X. H. Wu, C. P. Liu, K. F. Xu, *et al.* Reversal of hyperglycemia in diabetic rats by portal vein transplantation of islet-like cells generated from bone marrow mesenchymal stem cells. *World J Gastroenterol* 2007;**13**:3342–3349.

72 Y. Zhang, W. Shen, J. Hua, *et al.* Pancreatic islet-like clusters from bone marrow mesenchymal stem cells of human first-trimester abortus can cure streptozocin-induced mouse diabetes. *Rejuvenation Res* 2010;**13**:695–706.

73 S. M. Phadnis, M. V. Joglekar, M. P. Dalvi, *et al.* Human bone marrow-derived mesenchymal cells differentiate and mature into endocrine pancreatic lineage *in vivo*. *Cytotherapy* 2011;**13**:279–293.

74 E. Karaoz, A. Okcu, Z. S. Ünal, *et al.* Adipose tissue-derived mesenchymal stromal cells efficiently differentiate into insulin-producing cells in pancreatic islet microenvironment both *in vitro* and *in vivo*. *Cytotherapy* 2013;**15**:557–570.

75 M. G. Solari, S. Srinivasan, I. Boumaza, *et al.* Marginal mass islet transplantation with autologous mesenchymal stem cells promotes long-term islet allograft survival and sustained normoglycemia. *J Autoimmun* 2009;**32**:116–124.

76 M. Figliuzzi, R. Cornolti, N. Perico, *et al.* Bone marrow-derived mesenchymal stem cells improve islet graft function in diabetic rats. *Transplant Proc* 2009;**41**:1797–1800.

77 T. Ito, S. Itakura, I. Todorov, *et al.* Mesenchymal stem cell and islet co-transplantation promotes graft revascularization and function. *Transplantation* 2010;**89**:1438–1445.

78 B. Longoni, E. Szilagyi, P. Quaranta, *et al.* Mesenchymal stem cells prevent acute rejection and prolong graft function in pancreatic islet transplantation. *Diabetes Technol Ther* 2010;**12**:435–446.

79 H. Wu, D. Wen, R. I. Mahato. Third-party mesenchymal stem cells improved human islet transplantation in a humanized diabetic mouse model. *Mol Ther* 2013;**21**:1778–1786.

80 F. R. Li, X. G. Wang, C. Y. Deng, *et al.* Immune modulation of co-transplantation mesenchymal stem cells with islet on T and dendritic cells. *Clin Exp Immunol* 2010;**161**:357–363.

81 Y. H. Kim, Y. M. Wee, M. Y. Choi, *et al.* Interleukin (IL)-10 induced by $CD11b^+$ cells and IL-10-activated regulatory T cells play a role in immune modulation of mesenchymal stem cells in rat islet allografts. *Mol Med* 2011;**17**:697–708.

82 F. R. Li, C. Y. Deng, X. G. Wang, *et al.* The effect of co-transplantation of bone marrow-derived mesenchymal stem cells and islet on maturation and function of bone marrow-derived dendritic cells in recipient mice. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi* 2010;**26**:646–649 (in Chinese).

83 D. M. Xu, X. F. Yu, D. Zhang, *et al.* Mesenchymal stem cells differentially mediate regulatory T cells and conventional effector T cells to protect fully allogeneic islet grafts in mice. *Diabetologia* 2012;**55**:1091–1102.

84 D. M. Berman, M. A. Willman, D. Han, *et al.* Mesenchymal stem cells enhance allogeneic islet engraftment in nonhuman primates. *Diabetes* 2010;**59**:2558–2568.

85 T. Bhaiji, Z. L. Zhi, J. C. Pickup. Improving cellular function and immune protection via layer-by-layer nanocoating of pancreatic islet beta-cell spheroids cocultured with mesenchymal stem cells. *J Biomed Mater Res A* 2012;**100**:1628–1636.

86 N. E. Davis, L. N. Beenken-Rothkopf, A. Mirsoian, *et al.* Enhanced function of pancreatic islets co-encapsulated with ECM proteins and mesenchymal stromal cells in a silk hydrogel. *Biomaterials* 2012; **33**:6691–6697.

87 C. L. Rackham, P. K. Dhadda, P. C. Chagastelles, *et al.* Pre-culturing islets with mesenchymal stromal cells using a direct contact configuration is beneficial for transplantation outcome in diabetic mice. *Cytotherapy* 2013;**15**:449–459.

88 K. S. Park, Y. S. Kim, J. H. Kim, *et al.* Trophic molecules derived from human mesenchymal stem cells enhance survival, function, and angiogenesis of isolated islets after transplantation. *Transplant* 2010;**89**:509–517.

89 Y. Lu, X. Jin, Y. Chen, *et al.* Mesenchymal stem cells protect islets from hypoxia/reoxygenation-induced injury. *Cell Biochem Funct* 2010;**28**:637–643.

90 J. Zhao, S. He, C. Wang, *et al.* Protective effect of bone marrow mesenchymal stem cells on islets from hypoxia/reoxygenation-induced injury. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* 2013;**27**:910–915. (in Chinese).

91 C. D. Ceresa, R. N. Ramcharan, P. J. Friend, A. Vaidya. Mesenchymal stromal cells promote bowel regeneration after intestinal transplantation: myth to mucosa. *Transpl Int* 2013; **26**:e91–e93.

92 S. M. Doğan, S. Kılınç, E. Kebapçı, *et al.* Mesenchymal stem cell therapy in patients with small bowel transplantation: single center experience. *World J Gastroenterol* 2014;**20**:8215–8220.

CHAPTER 57

# The role of mesenchymal stromal cells in allogeneic hematopoietic stem cell transplantation

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## 57.1 The immunobiology of allogeneic hematopoietic stem cell transplantation

There are four main immunologic processes that are associated with allogeneic hematopoietic stem cell transplantation (HSCT), regardless of whether or not the hematopoietic stem cell (HSC) donor is a family member who is genotypically or phenotypically identical with the recipient for the human leukocyte antigens (HLAs) of the major histocompatibility complex, or is a living unrelated volunteer who is phenotypically matched for all or most of the HLAs, or whether an umbilical cord blood (UCB) unit is used; in the latter case there is more tolerance for the degree of HLA mismatching. The four immunologic process are graft rejection, graft-versus-host disease (GVHD), the graft-versus-leukemia effect, and the recipient's response to infection.

### 57.1.1 Graft rejection and late marrow failure

Graft failure is when the donor marrow fails to engraft at all due to rejection by recipient T cells that have survived the pretransplant chemoradiation or chemotherapy. This is often a fatal event, but there may be a role for mesenchymal stromal cells (MSCs) in it. Marrow failure refers to a graft that has taken initially but subsequently peters out. One reason is a low number of HSCs infused. This is sometimes treated with retransplantation.

### 57.1.2 Graft-versus-host disease

Acute GVHD is defined as that occurring within 100 days of HSCT. After that it is called chronic GVHD. Both are serious and both can be fatal. Acute GVHD is thought to be due to the release of lipopolysaccharide (exotoxin) secondary to organ and tissue damage from the pretransplant preparative regimen. This results in a cascade-like release of proinflammatory factors, such as tumor necrosis factor-α, interleukin (IL)-1, and IL-6, which recruit T cells in particular, which in turn mediate an immune attack on the recipient's tissues. This occurs even if the donor and recipient are HLA-matched and is due to recognition by the donor T cells, infused with the stem cell inoculum, of minor (or non-HLA) histocompatibility antigens present on the recipient's cells but not on those of the donor. It is thought that minor histocompatibility antigens drive T cell activation via the CD40–CD154, the CD80–CD28, and the CD86–CTLA4 interactions, inducing proliferation and differentiation of effector T cells. (CTLA4 is cytotoxic T-lymphocyte-associated protein 4, also known as CD152.) It occurs even though the recipient is routinely given immunosuppressive treatment such as cyclosporine and

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methotrexate to prevent it. Both acute and chronic GVHD usually require additional active immune suppressive medication, such as corticosteroids, and GVHD is itself immunosuppressive because it targets lymphoid organs such as the lymph nodes, thymus, and spleen in addition to the more clinically apparent target organs of skin, liver, and the gastrointestinal tract [1]. Thus, the allograft recipient is immune suppressed before the transplant due to conventional chemotherapy and further immune suppressed by the pretransplant regimen, the prophylactic drugs given to minimize GVHD, the immunosuppressive drugs given to treat GVHD, and GVHD itself.

### 57.1.3 The graft-versus-leukemia effect

The third immunologic process is the graft-versus-leukemia effect (or the graft-versus-malignancy effect if the recipient's diagnosis is a neoplasm other than leukemia). This is one of the major ways by which an allogeneic HSCT cures leukemia and other hematologic malignancies. As with GVHD, it too is mediated by donor T cells, and the respective populations are hard to separate. If relapse of the leukemia occurs it can sometimes be successfully treated simply by an infusion of lymphocytes from the marrow donor. Sensitivity to this procedure varies between different hematologic malignancies.

### 57.1.4 The recipient's response to infection

The fourth immunologic process is the recipient's response to infection. In view of the recipient's immune suppressed state it is not surprising that infections are common posttransplant. Prophylactic medication is routinely given with acyclovir, co-trimoxazole, an azole antifungal medication, and ganciclovir, to respectively minimize the risk of herpes simplex virus, *Pneumocystis jiroveci*, *Candida* and *Aspergillus* fungal infections, and cytomegalovirus. Happily, some elements of the innate immune system reconstitute quickly after the transplant. The most important of these is neutrophil recovery because of their importance in controlling bacterial diseases in conjunction with antibacterial antibiotics. The adaptive immune system, including both T cell and B cell function, reconstitutes much more slowly.

## 57.2 The immunobiology of mesenchymal stromal cells

The involvement of MSCs in both the innate and adaptive immune systems is multiple and complex and is described in Chapters 33 and 34.

## 57.3 The role of mesenchymal stromal cells in the expansion of hematopoietic stem cells

A major role of bone marrow mesenchymal stem and stromal cells is to support the function and survival of HSCs in the bone marrow. The two cell types are in close proximity to each in the marrow (Figure 57.1). The mechanisms underlying this are described in detail in Chapter 32.

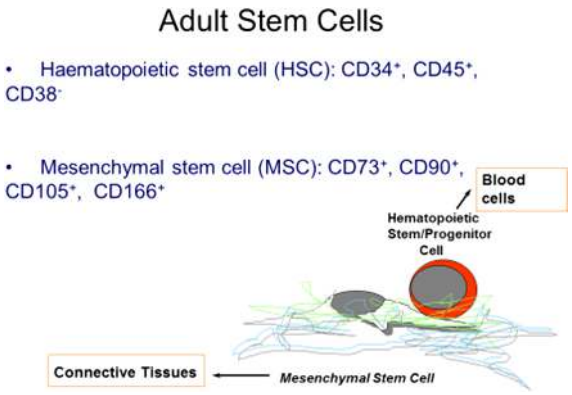



**Figure 57.1** HSCs are in close proximity to MSCs in the bone marrow and receive from them growth and differentiation signals. (See color plate section.)

This property of MSCs has been exploited clinically in patients undergoing UCB HSCT. While UCB HSCT works well in children, the number of HSCs present in one or two cord blood units is often insufficient to establish rapid or robust hematopoietic reconstitution in adults after UCB HSCT. After showing that expansion of both primitive and mature hematopoietic progenitors in unfractionated cord blood cells was markedly enhanced by co-culture with MSCs *in vitro*, de Lima *et al.* in Dr Shpall's group in Houston studied engraftment results in 31 adults with hematologic cancers who received transplants of two cord blood units, one of which contained cord blood that was expanded *ex vivo* in co-cultures with allogeneic MSCs [2]. The results in these patients were compared with those in 80 historical controls who received two units of unmanipulated cord blood. Co-culture with MSCs led to an expansion of total nucleated cells by a median factor of 12.2 and of $CD34^+$ cells by a median factor of 30.1. In patients in whom engraftment occurred, the median time to neutrophil engraftment was 15 days in the recipients of expanded cord blood compared with 24 days in controls who received unmanipulated cord blood only ($P < 0.001$). The median times to platelet engraftment were 42 days and 49 days respectively ($P = 0.03$). On day 26 posttransplant the cumulative incidence of neutrophil engraftment was 88% with expansion versus 53% without expansion ($P < 0.001$). On day 60 posttransplant the cumulative incidences of platelet engraftment were 71% and 31% respectively ($P < 0.001$). This was an encouraging result that needs confirmation in further trials.

## 57.4 The role of mesenchymal stromal cells in marrow graft rejection

Meuleman*et al.* evaluated the safety and efficiency of an infusion of donor MSCs that had been expanded in *ex vivo* culture in six patients with poor hematopoietic recovery after allogeneic HSCT [3]. The MSCs were infused without HSCs and without

conditioning at a dose of $1 \times 10^6$/kg weight. Two patients displayed rapid hematopoietic recovery on days 12 and 21 after the MSC infusion, while the other four patients showed no response. The two patients who showed hematopoietic recovery were in first complete remission, while the four who did not respond to MSCs had been more heavily pretreated. There were no toxic side effects from the MSC infusions. Thus, this small study of MSCs without HSC co-infusion suggests that MSCs can restore medullary function in some patients who have poor hematopoietic recovery after an allogeneic HSC transplant.

## 57.5 The role of mesenchymal stromal cells in the prevention of acute graft-versus-host disease

Three studies that included a randomization of MSCs or no MSCs to prevent GVHD after allogeneic HSCT have been reported [4–6]. Ning *et al.* performed a randomized clinical trial with 30 patients given marrow and/or peripheral blood stem cells from an HLA-identical sibling donor with ($n = 15$) or without ($n = 15$) added MSCs after myeloablative conditioning [4]. The authors compared the outcomes of the 10 evaluable patient given MSCs with those observed in the 15 patients randomized to the control arm. One of 10 patients in the MSC arm versus 8 of 15 in the control arm experienced grade II–IV acute GVHD. However, patients given MSCs had a higher risk of relapse (60% versus 20% at 3 years; $P = 0.02$), and lower disease-free survival (30% versus 67% at 3 years; $P = 0.035$) than those in the control arm, an outcome eerily reminiscent of T-cell-depleted allografts in the 1980s.

Bernardo *et al.* co-transplanted parental HLA-disparate MSCs in 13 children with hematological disorders undergoing unrelated cord blood transplantation after myeloablative conditioning [5]. HSCT outcomes were compared with those observed in a cohort of 39 matched historical controls. The cumulative incidence of graft rejection was 15% in the MSC group versus 3% in the historical group (not significantly different). The incidence of grade III–IV acute GVHD was 0% in the MSC group versus 26% in the historical group ($P = 0.05$), while the cumulative incidence of chronic GVHD was 0% in the MSC group versus 11% in the historical group (not significantly different). The 3-year overall survival was 63% and 64% in the MSC and control groups respectively, while the 3-year cumulative incidence of relapse was 25% and 23% respectively.

Baron *et al.* reported the results of a pilot study analyzing the safety of MSC co-transplantation in 20 patients given HLA-mismatched PBSCs after pretransplant conditioning with fludarabine and toal body irradiation [6]. MSCs were given at doses of 1–2 $\times$ $10^6$ MSCs/kg. Transplantation outcomes in these 20 patients were compared with those observed in a historical group of 16 patients given HLA-mismatched PBSCs (without MSCs) after the same nonmyeloablative conditioning. One of 20 patients in the MSC group versus 0 of 16 patients in the historical group experienced graft rejection. The 1- and 2-year probabilities of dying from GVHD or infection while on treatment for GVHD were 10% and 10% respectively in the MSC group, versus 31% and 38% respectively in the historical group ($P = 0.04$).

These three studies described lower risks of acute GVHD in patients co-transplanted with MSCs compared with historical or concurrent controls. However, the number of patients involved was relatively small. Large randomized, double-blinded, placebo-controlled studies are required to determine the potential utility of MSCs given as prophylaxis for GVHD while determining the effect, if any, on graft rejection, serious infection, and recurrence of the underlying hematological malignancy.

## 57.6 The role of mesenchymal cells in the treatment of corticosteroid-refractory acute graft-versus-host disease

One of the most dramatic single case reports in the history of allogeneic HSCT is of a 9-year-old boy with acute lymphoblastic leukemia in third remission who had received a transplant of blood stem cells from an HLA-A, HLA-B, HLA-DR-β1 identical, unrelated female donor after conditioning with cyclophosphamide (120 mg/kg) and fractionated total body irradiation (3 Gy for 4 days) [7]. Immunosuppression included thymoglobulin (6 mg/kg) during the conditioning, followed by cyclosporine combined with four doses of methotrexate. On day 11 after the allogeneic HSC transplant he developed a maculopapular rash of the thorax and back that progressed despite treatment with prednisolone 2 mg/kg daily. By day 22 the patient had developed diarrhea (>1000 mL/day) and abdominal pain requiring morphine. He stopped eating on day 24. Bilirubin and alanine aminotransferase concentrations rose. Psoralen and ultraviolet-A light (PUVA) treatment two to three times per week for 3 weeks was followed by extracorporeal PUVA one to four times per week for 6 weeks. Both infliximab (a monoclonal antibody to tumor necrosis factor-α) 10 mg/kg and daclizumab (a monoclonal antibody to the interleukin 2 receptor) 1 mg/kg for 4 weeks were ineffective. Methylprednisolone 250–500 mg/day for a total of 22 days, mycophenolate mofetil, and methotrexate were also tried. By day 70 the patient had developed grade IV (severe, usually fatal) acute GVHD, including diarrhea up to 20 times daily and a bilirubin concentration of 250 mmol/L. He was treated for repeated bacterial, viral, and invasive fungal infections. Because of their ability to suppress T cell alloproliferation it was decided to give maternal (haploidentical) MSCs. After 3 weeks of cell culture $90 \times 10^6$ MSCs were harvested. These cells expressed CD166, CD105, CD44, and CD29 but not CD34 or CD45. Intravenous administration of $2 \times 10^6$ MSCs/kg of the patient's weight were given on day 73. There was no toxicity after the infusion. Four days later the frequency of diarrhea fell to twice daily. A decline in total bilirubin was noted 5 days after the MSC infusion, and 2 weeks later the patient resumed oral food intake on day 84. By day 143 after the HSC allograft, DNA analysis of the

patient's bone marrow showed the presence of minimal residual leukemia. Cyclosporine treatment was discontinued to enable a graft-versus-leukemia effect. By day 150 the patient again had diarrhea but no abdominal pain. Colonoscopy showed normal colon with slight irritation of the cecum. Biopsy specimens showed mild GVHD and 4% female epithelium by fluorescence in situ hybridization. He received a transplant of the same batch of MSCs from his mother ($1 \times 10^6$/kg) on day 170 after transplantation. After 1 week his stools were normal and he started to eat again. His serum bilirubin concentration rose and liver biopsy specimens showed chronic GVHD. The bilirubin value subsequently returned to normal. He went home on day 220. He was well 1 year after transplantation, with a bilirubin concentration of 8 mmol/L. He had no minimal residual leukemia in his blood or bone marrow.

This was an astonishing case and stirred huge interest amongst marrow transplanters.

In a subsequent phase II trial in Europe, 55 patients were given MSCs at a median dose $1.4 \times 10^6$ MSCs/kg as treatment for corticosteroid-refractory grades II ($n = 5$), III ($n = 25$) or IV ($n = 25$) acute GVHD [8]. Thirty patients had complete responses and a further nine showed some improvement. Three patients had recurrence of their malignancy and one developed *de novo* acute myeloid leukemia of recipient origin. Complete responders to MSCs were more likely to be alive 2 years after HSCT than those with partial or no responses (52% versus 16%; ($P = 0.018$).

A phase III trial in North America undertaken by Osiris Therapeutics Inc. using unrelated donor bone-marrow-derived MSCs under the brand name Prochymal™ failed to reach its primary endpoints.

There appears little doubt that some patients with severe-corticosteroid-refractory acute GVHD respond to MSCs, particularly children, and Prochymal™ has marketing approval for its use in children from the regulatory authorities in Canada and New Zealand. The results of further phase II and III trials are awaited.

## 57.7 The mesenchymal stromal cell exosome: a substitute for the mesenchymal stromal cell?

Extracellular vesicles (EVs) are membrane vesicles that are released by many cell types into the extracellular space and consist of several classes, including exosomes, microvesicles, ectosomes, membrane particles, exosome-like vesicles, or apoptotic bodies. See Chapters 20 and 38 for details. However, the defining parameters for each of these different classes are not definitive, and the use of the terms exosomes, microvesicles, and microparticles are often ambiguous and not rigorously qualified. Generally, EVs can be formed by either inward budding of endolysosomal vesicles followed by exocytosis (e.g., exosomes) or shedding from the plasma membrane (e.g., microvesicles). They have attracted much interest from the biomedical research community because of their potential as sources of biomarkers for diseases, therapeutic agents, or vehicles for drug delivery. Generally, EVs carry a cargo of proteins and nucleic acids that reflects their cell of origin and the pathophysiological state of the cell source. In addition, these EVs with their cargoes could be internalized by other cells as a form of intercellular communication, making these vesicles potential therapeutic agents or delivery vehicles of therapeutic agents.

The exosome is the best characterized of all the EVs described to date and can be differentiated from other EVs by its biogenesis. The exosome is the only EV known to be derived from endosome, and this endosomal origin of exosomes is reflected in its membrane, which is enriched for lipids commonly found in lipid rafts such as cholesterol, sphingomyelin, and ceramide. Lipid rafts are specialized microdomains in the plasma membrane where much of the cellular endocytosis takes place to form endosomes. Subsequent invaginations of the endosomal membrane generate the numerous intraluminal vesicles within the multivesicular body (MVB). If the MVB fuses with the plasma membrane, the intraluminal vesicles are released as exosomes. The process by which cellular proteins and RNAs are selected and loaded into exosomes has not been elucidated. MSC exosomes have been among the best characterized exosomes to date (Chapters 1, 20, and 38).

Recently, MSC exosomes were administered to a person with treatment-refractory grade IV (severe) acute GVHD who went into remission with a dramatic alleviation of her disease symptoms and who remained stable for 5 months [9].

## References


1 DR Couriel. Diagnosis and treatment of graft-versus-host disease. In: Munker R, Hildebrandt GC, Lazarus HM, Atkinson K (eds), *The BMT Data Book*, third edition. Cambridge University Press: Cambridge, 2013; pp. 311–329.

2 M de Lima, I McNiece, SN Robinson, *et al.* Cord-blood engraftment with *ex vivo* mesenchymal-cell coculture. *N Engl J Med* 2012;**367**: 2305–2315.

3 N Meuleman, T Tondreau, I Ahmad, *et al.* Infusion of mesenchymal stromal cells can aid hematopoietic recovery following allogeneic hematopoietic stem cell myeloablative transplant: a pilot study. *Stem Cells Dev* 2009;**10**:1247–1252.

4 H Ning, F Yang, M Jiang, *et al.* The correlation between cotransplantation of mesenchymal stem cells and higher recurrence rate in hematologic malignancy patients: outcome of a pilot clinical study. *Leukemia* 2008;**22**:593–599.

5 ME Bernardo, LM Ball, AMCometa, *et al.* Co-infusion of *ex vivo*-expanded, parental MSCs prevents life-threatening acute GVHD, but does not reduce the risk of graft failure in pediatric patients undergoing allogeneic umbilical cord blood transplantation. *Bone Marrow Transplant* 2011;**46**:200–207.

6 F Baron, C Lechanteur, E Willems, *et al.* Cotransplantation of mesenchymal stem cells might prevent death from graft-versus-host disease (GVHD) without abrogating graft-versus-tumor effects after HLA-

mismatched allogeneic transplantation following nonmyeloablative conditioning. *Biol Blood Marrow Transplant* 2010;**16**: 838–847.

7 K Le Blanc, I Rasmusson, B Sundberg, *et al*. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–1441.

8 K Le Blanc, F Frassoni, L Ball, *et al*. Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**:1579–1586.

9 L Kordelas, V Rebmann, AK Ludwig, *et al*. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 2014;**28**:970–973.

CHAPTER 58

# The role of mesenchymal stromal cells in the management of skin wounds

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## 58.1 Introduction

Chronic skin wounds and wounds associated with disease remain challenging clinical problems in aging and westernized societies [1]. These wounds do not always respond to current treatments [2]. Recently, cell therapy has emerged as a new therapeutic approach for wound care, and much effort has focused on understanding its therapeutic mechanism. In particular, mesenchymal stromal cells (MSCs) are one of the most readily available sources of cells, showing obvious beneficial effects in wound healing [3]. Recent reports also highlighted the beneficial properties of placental-tissue-derived MSCs in wounds [4,5]. This review discusses the critical roles of MSCs in the wound healing process.

## 58.2 The wound healing process

The definition of a skin wound is damage or disruption to the normal anatomical structure and function, ranging from a simple break in subcutaneous tissue to deep damage to other structures, such as muscles, nerves, and vessels [6,7]. Wounds can be classified as acute or chronic depending on the healing time [8,9]. Acute wounds are those that proceed through normal stages of the healing pathway, with eventual functional and anatomical restoration. Usual healing time may last up to 30 days. Chronic wounds, on the other hand, cannot be repaired in a timely and orderly manner by the normal healing process due to various causes, including arterial and venous insufficiency [6,10]. The healing process is disturbed by many factors, such as necrosis, continuous state of inflammation, hypoxia, and infection. Another type of wound is a complicated wound, defined as a combination of tissue defect and infection [9]. The five signs and symptoms of edema, heat, pain, redness, and limited function are typical properties. The wound healing process is a complex and dynamic event involving a series of coordinated interactions in biological and immunological systems. Various cell types and a cascade of regulated steps are involved in wound healing [11]. These processes occur in four overlapping, time-dependent phases: coagulation/hemostasis, inflammation, proliferation, and wound remodeling [12]. Immediately after injury, coagulation and hemostasis take place in the wound to protect the vascular system and minimize blood loss. Platelets and blood clots contain fibrin, fibronectin, vitronectin, and various growth factors, such as insulin-like growth factor and transforming growth factor (TGF)-β, that act as promoters for the healing cascade by activation and attraction of neutrophils, fibroblasts, macrophages, and endothelial cells [13]. During the coagulation/hemostasis phase, the inflammation phase begins with infiltration of neutrophils in the wound site 1 day after injury to prevent infection [13]. Neutrophils play an important role in removing and destroying bacteria, foreign particles, dead cells, and damaged extracellular matrix by phagocytic activity. Upon finishing this task, neutrophils are eliminated from the wound area, and macrophages phagocytose redundant and apoptotic cells [14]. About 2 days after injury, macrophages derived from blood

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monocytes continue the late inflammatory phase. These cells regulate and activate keratinocytes, fibroblasts, and endothelial cells by providing an abundant reservoir of various tissue growth factors, such as TGF-β, fibroblast growth factor (FGF), epidermal growth factor, and collagenase [15]. About 3 days after injury, lymphocytes are attracted to the wound site and work by activation of interleukin (IL)-1 and immunoglobulin G breakdown [16]. IL-1 is responsible for collagen regulation, remodeling, and degradation [17]. About 2 weeks thereafter, the proliferative phase starts through fibroblast migration, deposition of extracellular matrix, and formation of granulation tissue [7]. Migrated fibroblasts and myofibroblasts are stimulated to proliferate and produce matrix protein. The resulting abundant extracellular matrix supports cell migration, and fibroblasts change into the myofibroblast phenotype. Another important factor for wound healing is the collagen synthesized by fibroblasts. It plays a pivotal role in the proliferative and remodeling phases by providing a foundation for intracellular matrix formation [18,19]. In addition, numerous angiogenic factors establish new blood vessels, which are critical for all phases of wound healing. Vascular endothelial growth factor (VEGF) and FGF are representative regulatory factors in all reparative processes. Changes in wound microenvironment, such as hypoxia, low pH, and increased lactate, also initiate and promote angiogenesis [20]. Chemotactic agents play a major role in angiogenesis, cell migration, and modulation of cell growth and differentiation [21]. The last phase of wound healing is the remodeling phase, which is responsible for the formation of new epithelium and final scar tissue. This phase usually lasts for 1 or 2 years but sometimes may last longer. Wound remodeling is controlled by a delicate balance of synthesis and degradation. Along with intracellular matrix maturation, collagen bundles increase, and fibronectin and hyaluronic acid are degraded [18]. Tissue inhibitor of metalloproteinase activity and tensile strength increase with collagen collection, and new matrix accumulates [22]. New collagen matrix and its subsequent organization are completed in the remodeling phase. At the same time, wound contraction begins, and the density of macrophages and fibroblasts is reduced. Finally, metabolic activity decreases, capillary growth stops, and the full wound healing process is completed with a mature scar.

## 58.3 The role of mesenchymal stromal cells in the wound healing process

MSCs play an important role in all phases of wound healing, especially the inflammation, proliferation, and remodeling processes.

### 58.3.1 Immune modulation

MSCs directly attenuate the inflammatory response by decreasing secretion of interferon-γ and TNF-β, two representative proinflammatory cytokines. In addition, MSCs produce anti-inflammatory cytokines such as IL-10, IL-4, and TGF-β [23,24]. Recent studies have also revealed that MSCs secrete TNF-stimulated gene-6, an anti-inflammatory factor that suppresses TNF-α-mediated inflammation, excessive collagen deposition, and myofibroblast differentiation [25]. These properties of MSCs influence the inflammatory phase and the chronic wound state.

### 58.3.2 Antimicrobial activity

Antimicrobial activity is critical for wound clearance of infection. The antimicrobial activity of MSCs is mediated by two mechanisms: secretion of LL-37, an antimicrobial factor [26], and secretion of immunomodulating factors that regulate immune cells and increase phagocytosis and bacterial killing [27] (and see Chapter 55).

### 58.3.3 Chemotactic and migratory activities

Chemotactic signals modulate inflammation and cause MSCs to migrate to wound sites and facilitate tissue regeneration [28]. MSCs secrete high levels of chemokines, such as macrophage inflammatory protein (MIP)-1α, MIP-2, monocyte chemoattractant protein 5, granulocyte chemotactic protein 2, neutrophil-activating protein 2, IL-8, chemokine receptor (CCR)2, CCR3, and CCR5 [29,30]. Secreted factors from MSCs recruit macrophages and endothelial lineage cells [29]. In addition, MSCs show high migration and proliferation activity using chemotactic and angiogenic factors to promote wound healing [4,29,30].

### 58.3.4 Paracrine activity

Paracrine signaling associated with angiogenesis improves tissue repair and is likely the main mechanism of wound healing. Many paracrine factors of MSCs have been reported such as angiopoietin-1, VEGF, FGF, epidermal growth factor, platelet-derived growth factor, TGF-β, and keratinocyte growth factor [4,31,32]. These paracrine signaling molecules have anti-scarring properties through hepatocyte growth factor, VEGF, TGF-β1, and TGF-β3 [33,34].

### 58.3.5 Differentiation

Differentiation of MSCs has also been implicated as one mechanism of wound healing. In fact, human MSCs survived for more than 1 year in wounded tissues of a fetal lamb model [35]. In addition, MSCs differentiated into keratinocytes in the wound site, indicating direct contribution to cutaneous regeneration [4,31,36]. Close examination revealed that injected MSCs were found in dermal appendages [31]. MSCs differentiate into several cell types in the wound, such as endothelial cells, pericytes, and monocytes [37,38]; however, low engraftment and survival of MSCs at wound sites may cause limited activity of MSC differentiation.

## 58.4 Conclusions and the future

Applying MSCs to skin wound healing appears to have obvious beneficial effects. Even allogeneic MSCs exhibit minimal

immunogenicity and are equally efficient as syngeneic MSCs in engraftment and enhancement of wound healing [39]. Their limited plasticity and varied potential for paracrine signaling from different cell sources or tissues may influence their therapeutic efficacy in the wound healing process. The physiological and molecular mechanisms of MSCs in wound healing are complicated. Thus, cutting-edge technology should be used to elucidate further details of MSC-mediated mechanisms in different wound healing phases.

## References


1 Natarajan S, Williamson D, Stiltz AJ, Harding K. Advances in wound care and healing technology. *American Journal of Clinical Dermatology* 2000;**1**(5):269–275.

2 Mustoe TA, O'Shaughnessy K, Kloeters O. Chronic wound pathogenesis and current treatment strategies: a unifying hypothesis. *Plastic and Reconstructive Surgery* 2006;**117**(7 Suppl): 35S–41S.

3 Da Silva Meirelles L, Caplan AI, Nardi NB. In search of the *in vivo* identity of mesenchymal stem cells. *Stem Cells* 2008;**26**(9): 2287–2299.

4 Kim SW, Zhang HZ, Guo L, *et al*. Amniotic mesenchymal stem cells enhance wound healing in diabetic NOD/SCID mice through high angiogenic and engraftment capabilities. *PLoS ONE* 2012;**7** (7):e41105.

5 Mermet I, Pottier N, Sainthillier JM, *et al*. Use of amniotic membrane transplantation in the treatment of venous leg ulcers. *Wound Repair and Regeneration* 2007;**15**(4):459–464.

6 Robson MC, Steed DL, Franz MG. Wound healing: biologic features and approaches to maximize healing trajectories. *Current Problems in Surgery* 2001;**38**(2):72–140.

7 Velnar T, Bailey T, Smrkolj V. The wound healing process: an overview of the cellular and molecular mechanisms. *The Journal of International Medical Research* 2009;**37**(5):1528–1542.

8 Lazarus GS, Cooper DM, Knighton DR, *et al*. Definitions and guidelines for assessment of wounds and evaluation of healing. *Archives of Dermatology* 1994;**130**(4):489–493.

9 Bischoff M, Kinzl L, Schmelz A. The complicated wound. *Der Unfallchirurg* 1999;**102**(10):797–804 (in German).

10 Degreef HJ. How to heal a wound fast. *Dermatologic Clinics* 1998;**16** (2):365–375.

11 Attinger CE, Janis JE, Steinberg J, *et al*. Clinical approach to wounds: debridement and wound bed preparation including the use of dressings and wound-healing adjuvants. *Plastic and Reconstructive Surgery* 2006;**117**(7 Suppl):72S–109S.

12 Vanwijck R. Surgical biology of wound healing. *Bulletin et Memoires de l'Academie Royale de Medecine de Belgique* 2001;**156** (3–4):175–184, discussion 185 (in French).

13 Broughton II G, Janis JE, Attinger CE. The basic science of wound healing. *Plastic and Reconstructive Surgery* 2006;**117**(7 Suppl):12S-34S.

14 Diegelmann RF, Evans MC. Wound healing: an overview of acute, fibrotic and delayed healing. *Frontiers in Bioscience* 2004;**9**:283–289.

15 Ramasastry SS. Acute wounds. *Clinics in Plastic Surgery* 2005;**32** (2):195–208.

16 Hunt TK, Hopf H, Hussain Z. Physiology of wound healing. *Advances in Skin & Wound Care* 2000;**13**(2 Suppl):6–11.

17 Sieggreen MY. Healing of physical wounds. *The Nursing Clinics of North America* 1987;**22**(2):439–447.

18 Baum CL, Arpey CJ. Normal cutaneous wound healing: clinical correlation with cellular and molecular events. *Dermatologic Surgery* 2005;**31**(6):674–686, discussion 686.

19 Clark RA. Regulation of fibroplasia in cutaneous wound repair. *The American Journal of the Medical Sciences* 1993;**306**(1):42–48.

20 Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. *The Journal of Investigative Dermatology Symposium Proceedings* 2000;**5**(1):40–46.

21 Hsu S, Thakar R, Li S. Haptotaxis of endothelial cell migration under flow. *Methods in Molecular Medicine* 2007;**139**:237–250.

22 Mulder GD, Vande Berg JS. Cellular senescence and matrix metalloproteinase activity in chronic wounds. Relevance to debridement and new technologies. *Journal of the American Podiatric Medical Association* 2002;**92**(1):34–37.

23 Aggarwal S, Pittenger MF. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.

24 Oh JY, Kim MK, Shin MS, *et al*. The anti-inflammatory and anti-angiogenic role of mesenchymal stem cells in corneal wound healing following chemical injury. *Stem Cells* 2008;**26**(4):1047–1055.

25 Qi Y, Jiang D, Sindrilaru A, *et al*. TSG-6 released from intradermally injected mesenchymal stem cells accelerates wound healing and reduces tissue fibrosis in murine full-thickness skin wounds. *The Journal of Investigative Dermatology* 2014;**134**(2):526–537.

26 Krasnodembskaya A, Song Y, Fang X, *et al*. Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. *Stem Cells* 2010;**28**(12):2229–2238.

27 Mei SH, Haitsma JJ Dos Santos CC, *et al*. Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. *American Journal of Respiratory and Critical Care Medicine* 2010;**182**(8):1047–1057.

28 Newman RE, Yoo D, LeRoux MA, Danilkovitch-Miagkova A. Treatment of inflammatory diseases with mesenchymal stem cells. *Inflammation & Allergy Drug Targets* 2009;**8**(2):110–123.

29 Chen L, Tredget EE, Wu PY, Wu Y. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3**(4):e1886.

30 Kim SW, Zhang HZ, Kim CE, *et al*. Amniotic mesenchymal stem cells with robust chemotactic properties are effective in the treatment of a myocardial infarction model. *International Journal of Cardiology* 2013;**168**(2):1062–1069.

31 Wu Y, Chen L, Scott PG, Tredget EE. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* 2007;**25**(10):2648–2659.

32 Kim SW, Zhang HZ, Kim CE, *et al*. Amniotic mesenchymal stem cells have robust angiogenic properties and are effective in treating hindlimb ischaemia. *Cardiovascular Research* 2012;**93**(3):525–534.

33 Ono I, Yamashita T, Hida T, *et al*. Combined administration of basic fibroblast growth factor protein and the hepatocyte growth factor gene enhances the regeneration of dermis in acute incisional wounds. *Wound Repair and Regeneration* 2004;**12**(1):67–79.

34 Shah M, Foreman DM, Ferguson MW. Neutralisation of TGF-β1 and TGF-β2 or exogenous addition of TGF-β3 to cutaneous rat wounds reduces scarring. *Journal of Cell Science* 1995;**108**(Pt 3): 985–1002.

35 Mackenzie TC, Flake AW. Human mesenchymal stem cells persist, demonstrate site-specific multipotential differentiation, and are present in sites of wound healing and tissue regeneration after transplantation into fetal sheep. *Blood Cells, Molecules & Diseases* 2001;**27**(3):601–604.

36 Nakagawa H, Akita S, Fukui M, *et al*. Human mesenchymal stem cells successfully improve skin-substitute wound healing. *The British Journal of Dermatology* 2005;**153**(1):29–36.

37 Sasaki M, Abe R, Fujita Y, *et al*. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *Journal of Immunology* 2008;**180**(4):2581–2587.

38 Shumakov VI, Onishchenko NA, Rasulov MF, *et al*. Mesenchymal bone marrow stem cells more effectively stimulate regeneration of deep burn wounds than embryonic fibroblasts. *Bulletin of Experimental Biology and Medicine* 2003;**136**(2):192–195.

39 Chen L, Tredget EE, Liu C, Wu Y. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. *PLoS ONE* 2009;**4**(9):e7119.

CHAPTER 59

# The role of mesenchymal stromal cells in skin wound healing

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## 59.1 Summary

It is becoming increasingly evident that adult stromal cells have the capacity to participate in repair and regeneration of damaged and/or diseased tissues. Mesenchymal stromal cells (MSCs) have been among the most studied adult stromal cells for the treatment of a variety of conditions, including wound healing. Wound healing is a complex multistage process that orchestrates the reconstitution of the dermal and epidermal layers of the skin. Owing to the recent advances in stromal cell research, enthusiasm has arisen for the application of stromal cells in regenerative medicine. MSCs have been shown to improve skin wound healing in multiple preclinical models and in a few clinical studies. In this chapter we will summarize approaches for improving wound healing and outline mechanisms determined in preclinical studies. Future directions of MSC-based therapy in wound healing will also be explored. Finally, despite its apparent advantages, the limitations and challenges of MSC administration to improve wound healing will be discussed.

## 59.2 Introduction

A wound is defined as a disruption of normal anatomic structure and functional integrity of the skin or another external surface [1]. In many pathological circumstances, such as diabetes or burns injury, the normal wound healing process fails to adequately restore function to the skin, leading to potentially severe complications, including ulcers and infections. Normal wound healing is a dynamic and complex process involving a series of coordinated events, including bleeding and coagulation, acute inflammation, cell migration, proliferation, differentiation, angiogenesis, re-epithelialization, and synthesis and remodeling of the extracellular matrix (ECM) [2].

Mesenchymal stromal cells (MSCs) orchestrate the three main phases of normal wound healing: inflammatory, proliferative, and remodeling. MSCs directly attenuate the inflammatory response. They also promote antimicrobial activity (see Chapter 55) and cell migration during epithelial remodeling [2] (and see Chapters 22, 23, and 24).

The procurement, isolation, expansion, and cryostorage of MSCs is relatively straightforward [3]. MSCs can be derived from many different organs and tissues including bone marrow, adipose tissue, amniotic fluid, umbilical cord, placenta, menstrual blood, and dental pulp [4–7]. MSCs secrete many cytokines and chemokines that are antiapoptotic, immunomodulatory and proangiogenic [8].

## 59.3 The role of bone-marrow-derived mesenchymal stromal cells in wound healing

Bone-marrow-derived MSCs (BM-MSCs) improved cutaneous healing by accelerating re-epithelialization, increasing angiogenesis,

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and differentiating into epithelial cells expressing the keratinocyte-specific marker keratin [9]. The trophic (supportive) activity of MSCs may include both dermal fibroblast and keratinocyte migration, as well as contribute to the formation of the ECM [10]. Preclinical evidence supports the therapeutic application of BM-MSCs as summarized in Table 59.1.

Stoff *et al.* reported that the injection of human BM-MSCs into an incisional wound led to improved wound closure and a significant reduction in scar formation in rabbits. Incisional wounds treated with human MSCs regained tensile strength at a significantly higher level than untreated wounds did [11]. Murine BM-MSCs applied to full-thickness excisional wounds

**Table 59.1** Preclinical studies using adult mesenchymal stromal cells to enhance cutaneous wound healing.

| Cell type | Wound model | Treatment method | Outcome | Reference |
|---|---|---|---|---|
| BM-MSCs | Rabbit model of full-thickness 3 cm long incisional wounds up to the fascia in the dorsal skin | Intradermal injection of human MSCs | Reduced wound scarring and increased tensile strength in the incisional wounds | [11] |
| | Mouse full-thickness excisional wound | Intradermal injection of either allogeneic or syngeneic murine BM-MSCs | Migration through the tissue, attenuation of the local inflammatory response, and facilitation of cutaneous healing was similar with either allogeneic or syngeneic MSCs | [12] |
| | Human fibrin clot in a wounded tissue | *In vitro* culture system of fibrin clot and human MSCs | A high intrinsic fibrinolytic capacity of human MSCs was found which mediates the invasion into a fibrin clot in a wounded tissue | [13] |
| | Cutaneous wounds in diabetic (db/db) mice | Murine BM-MSCs were administered intradermally around the wound edge together with the novel reparative lipid mediator 14*S*,21*R*-dihydroxydocosa-hexaenoic acid | Accelerated wound re-epithelialization, granulation tissue formation, and improved vascularization | [14] |
| | Minipig model of local exposure to high doses of γ-gamma radiation (50 Gy) | Intradermal injection of autologous BM-MSCs | Accumulation of lymphocytes at the border of the dermis and subcutaneous tissue; improved vascularization | [15] |
| | A single dorsal full- thickness wound with an 8 mm punch biopsy in diabetic (db/db) mice | Subcutaneous injection of syngeneic BM-MSCs | Increased miR-146a expression, attenuated inflammatory gene expression, and improved diabetic wound healing | [16] |
| | 1.5 cm dorsal skin incision in B6D2 mice | Sutures were co-cultured in the presence of B6D2 BM-MSCs | Increased the collagen deposition in sutured wounds | [17] |
| | 24 full-thickness circular wounds 6 mm in diameter in beagle dogs | Topical application of allogeneic BM-MSCs | More rapid wound closure, increased collagen synthesis, cellular proliferation, and angiogenesis | [18] |
| | Excisional wounds in C57BL/6 mice | Subcutaneous injection at four equidistant points around the wound edge of murine MSCs seeded on hydrogels | MSC-seeded hydrogels demonstrated significantly enhanced angiogenesis compared with MSCs administered without a hydrogel scaffold | [19] |
| | Immunodeficient NOD/SCID mice were locally irradiated to the leg (30 Gy, dose rate 2.7 Gy/min) using a $^{60}$Co source to induce a severe skin lesion | Cultured human BM-MSCs were injected intravenously | Less severe lesions and more rapid healing of wounds compared with control mice | [20] |
| Adipose-tissue-derived MSCs (ADSCs) | Excisional wounds in diabetic (db/db) mice | Autologous ADSCs containing a telocollagen matrix with a silicone membrane on to an open wound | Wound healing promoted possibly due to cytokines secreted by ADSCs | [21] |
| | Full-thickness skin wounds in nude BALB/c mice | Intradermal injection of human ADSCs at bilateral sites along the periphery of the wound | Accelerated wound healing | [22] |
| | Porcine full-layer excisional wound model | Autologous or allogeneic porcine ADSCs administered in a fibrin gel vehicle containing a number of dynamic wound-healing trophic factors on to an open wound | The application of more than one growth factor together with MSCs demonstrated an advantage in improving wound healing | [23] |

**Table 59.1** (*Continued*)

| Cell type | Wound model | Treatment method | Outcome | Reference |
|---|---|---|---|---|
| | Mouse full-thickness excisional wounds | Intradermal injection around the wound at four injection sites of syngeneic murine BM-MSCs | Significantly higher capillary densities than control group | [24] |
| | Nude mouse full-thickness excisional wounds | Intradermal injection of human ADSCs | Conditioned medium (CM) from TGF-β1-pretreated ADSCs promoted wound healing | [25] |
| | Mouse full-thickness excisional wounds | Direct implantation of human ADSC sheets on to the wounds | Wound healing enhanced | [26] |
| Gestational-tissue-derived MSCs | Full-thickness skin excision wounds in SCID mice | Human Wharton's jelly MSCs injected into the skin seeded on to an amniotic membrane scaffold | Demonstrated best biomechanical properties; augmented scar-free wound healing with hair growth | [27] |
| | Rat model of 30% total body surface area full-thickness burns | Intravenous injection of green fluorescent protein-labeled human umbilical cord MSCs (UC-MSCs) | Promoted wound recovery, neovascularization, and re-epithelialization | [28] |
| | Rat 8 mm full-thickness circular excisional wounds | Intradermal injection around the wound area of human UC-MSCs | Promoted wound closure and increased new capillary formation | [29] |
| | Mouse full-thickness excisional skin wounds | Subcutaneous injection of hypoxia-conditioned medium from human amniotic-fluid-derived MSCs around the wound and topical application to the wound bed | Enhanced wound healing via an increase of hypoxia-induced paracrine factors | [30] |
| Dermal-tissue-derived MSCs | Murine bone marrow cells from enhanced green fluorescent protein transgenic mice were transplanted into normal C57BL/6 mice and into mice with excisional skin wounds | 1.5 $cm^2$ full-thickness wounds were made that included the panniculus carnosus muscle on the mid back | Normal skin was a target organ for bone-marrow-derived cells from both the hematopoietic and the mesenchymal stem cell pools. MSCs contributed more to wound healing than $CD45^+$ hematopoietic cells | [31] |

exhibited an ability to control allogeneic fibroblasts to migrate through the tissue and to attenuate the local inflammatory response. BM-MSC-treated wounds had significantly reduced $CD45^+$ leukocytes, $CD3^+$ T cells, and $CD8^+$ T cells [12].

An intrinsic fibrinolytic capacity of human MSCs mediated the invasion of cells into the fibrin clot of a wounded tissue. The secretion of the fibrinolytic enzymes tissue plasminogen activator, urokinase-type plasminogen activator, soluble urokinase-type plasminogen activator receptor (CD87), and plasminogen activator inhibitor by human MSCs resulted in fibrin clot degradation *in vitro*. Human MSCs and human dermal fibroblasts were both critically involved in remodeling the ECM in skin wounds [13].

14*S*,21*R*-dihydroxydocosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid (14*S*,21*R*-diHDHA) is a new agent used for treating diabetic skin ulcers. 14*S*,21*R*-diHDHA triggered phosphorylation of p38 but had no effect on phosphorylation of extracellular regulated kinase (ERK)1/2, suggesting that diabetes mellitus may affect the generation of pro-healing lipid mediators for restoring wound healing and MSC functions [14]. However, the mechanisms contributing to the healing process vary with BM-MSCs. Activation of adenosine and adenosine A2A receptors and CD73 coordinately regulate the proliferation and differentiation of BM-MSCs in knockout mice [32]. In addition hypoxia-dependent migration has a biphasic sequence involving an early phase requiring the generation of reactive oxygen species, and a delayed phase sustained by hypoxia-inducible-factor-1-dependent expression and release of vascular endothelial growth factor (VEGF) [33].

Platelet lysate is involved in the initiation of wound healing. An inflammatory response is induced in MSCs followed by the secretion of factors maintaining macrophages in a proinflammatory state, thus enhancing the initial inflammatory response to the injury. In an inflammatory microenvironment, platelet lysate also increased the secretion of the proinflammatory cytokine interleukin (IL)-6 [34]. Secreted frizzled-related protein 2 (sFRP2) has recently been identified as a key mediator of MSC-driven repair of myocardial injury and of wound repair. sFRP2 activity occurred because of inhibition of both wingless-related integration site (Wnt) and bone morphogenetic protein signaling pathways. Bone morphogenetic protein signaling inhibition mediated by sFRP2, as assessed by levels of pSMAD1/5/8, was independent of its effects on the Wnt pathway [35].

In a rat skin burn model MSC-derived exosomes are involved in tissue injury repair. MSC-exosome-derived Wnt4 promoted α-catenin nuclear translocation and activity to promote re-epithelialization and cell proliferation, a potential new mechanism for the use of MSCs in cutaneous wound healing [36].

A new minipig model has been developed that is close to humans and enables development of mesenchymal stromal cell therapy strategies that can be applied to the treatment of human radiation burns. In grafted animals MSCs led to local accumulation of lymphocytes at the border of the dermis and the subcutaneous tissue and improved vascularization [15].

Increasing evidence shows that MSCs are also strongly influenced by their biomechanical environment (see Chapter 17). Experiments in both animal and *in vitro* model systems have demonstrated pivotal roles for the biomechanical environment in regulating myofibroblast differentiation and contraction, endothelial barrier function and angiogenesis, and MSC fate decisions. In xenograft wound healing models these *in vitro* differences correlated with decreased ability of human MSCs to aid in wound closure *in vivo* [37]. Molecular mechanisms transducing the biomechanical environment are emerging, with prominent and interacting roles recently identified for key network components, including transforming growth factor-β (TGF-β)/SMAD, focal adhesion kinase, myocardin-related transcription factors, Wnt/β-catenin and Yes-associated protein (YAP)/TAZ (transcriptional coactivator with PDZ-binding motif) signaling pathways [38]. (Myocardin-related transcription factors transduce cytoskeletal signals to the nucleus, activating a subset of serum response factor-dependent genes promoting myogenic differentiation and cytoskeletal organization. They are multifunctional proteins that share evolutionarily conserved domains required for actin-binding, homo- and heterodimerization, high-order chromatin organization, and transcriptional activation. The Yorkie-homologues YAP and TAZ act as relays of mechanical signals exerted by ECM rigidity and cell shape.)

MSCs were used for skin wounds in a rat model of streptozotocin-induced diabetes mellitus. Histologic analysis showed a decrease in $CD45^+$ leukocytes in the MSC group compared with the control group. Significant increases in epidermal growth factor, VEGF, prolyl 4-hydroxylase, and Ki-67 expression were also noted in the MSC-treated group compared with the control group [39].

Xu *et al.* provided the first evidence that decreased expression of miR-146a in diabetic skin wounds may in part be responsible for the abnormal inflammatory response seen in diabetic skin wounds and may contribute to impaired wound healing. miR-146a expression was significantly reduced during the healing of diabetic skin wounds and was associated with a dramatic increase in the proinflammatory target genes IL-1-receptor-associated kinase 1, tumor necrosis factor (TNF)-receptor-associated factor 6, and nuclear factor kappa light chain-enhancer of activated B cells (NF-κB), resulting in increased gene expression of IL-6 and macrophage inhibitory protein 2 [16].

**Table 59.2** Clinical trials of cell-based therapy for wound healing registered on ClinicalTrials.gov.

| Disease | Cells used | Study phase | Results | Reference |
|---|---|---|---|---|
| Chronic wounds | BM-MSCs | Phase I | This study is ongoing but not recruiting | NCT01751282 |
| Diabetic foot ulcers | Autologous BM-MSCs | Phases I and II | Not yet recruiting | NCT01686139 |
| Diabetic critical limb ischemia | Tissue repair cells and BM-MSCs | Phase II | Completed | NCT01065337 |
| Diabetic foot ulcers, leg ulcers and ischemic ulcers | Autologous bone marrow cell concentrate | Phases II and III | Ongoing but not recruiting | NCT00434616 |
| Type 1 and type 2 diabetes mellitus with skin ulcers | Autologous BM-MSCs | Phases I and II | Not yet recruiting | NCT01686139 |
| Type 2 diabetes mellitus | Autologous ADSCs | Phases I and II | Unknown | NCT00703612 |
| Type 1 diabetes mellitus | Autologous ADSCs | Phases I and II | Unknown | NCT00703599 |
| Diabetic foot ulcers, venous ulcers, pressure ulcers | ADSCs | Phase II | Recruiting | NCT02092870 |
| Diabetes mellitus type 2 skin ulcers | Harvesting and implantation of ADSCs | Phases I and II | Recruiting | NCT01453751 |
| Diabetic foot ulcers and critical limb ischemia | UC-MSCs | Phases I and II | Unknown | NCT01216865 |
| Type 2 diabetes mellitus skin ulcers | UC-MSCs | Phases I and II | Recruiting | NCT01413035 |
| Burns | Human UC-MSCs | Phases I and II | Recruiting | NCT01443689 |
| Diabetic skin ulcers and venous stasis wounds | Lipoaspirate | Phase I | Unknown | NCT00815217 |
| Diabetic critical limb ischemia and foot ulcer | Autologous MSCs and mononuclear cells | Phase I | Completed. No study results | NCT00955669 |
| Epidermolysis bullosa | Umbilical cord blood cells | Phase II | Recruiting | NCT01033552 |

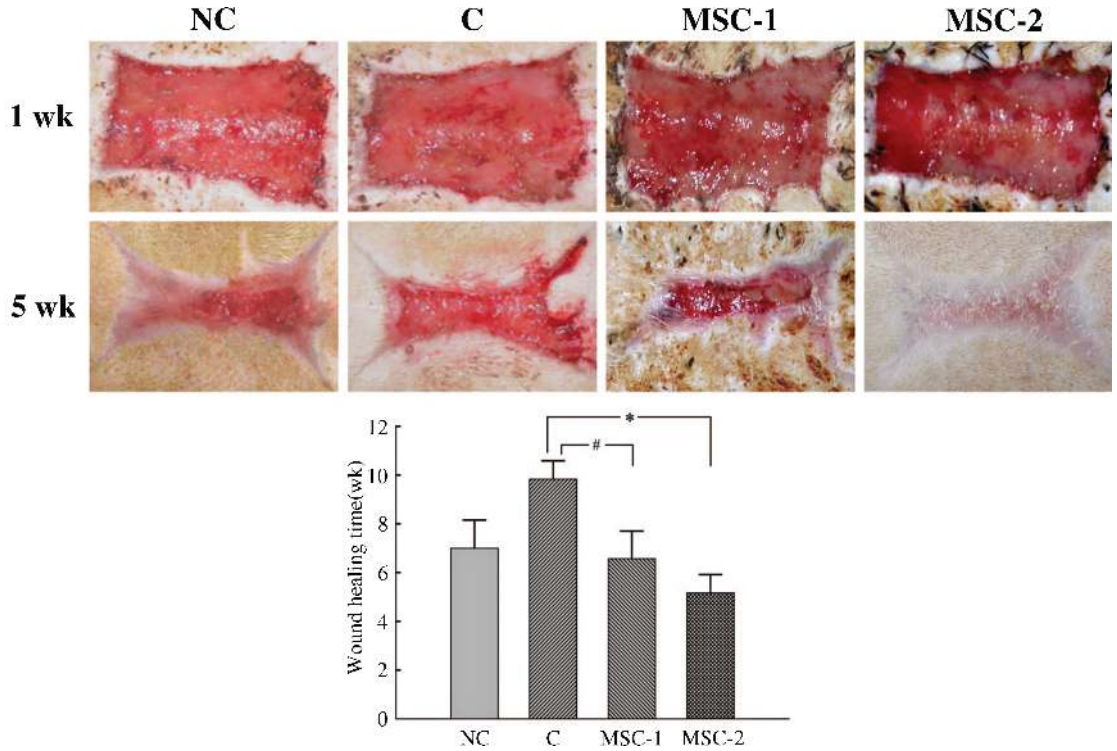



**Figure 59.1** MSC injection into the edge of the wound decreased the total time required for wound healing in diabetic rats. The wound size was significantly reduced in rats receiving MSC-1 (# $P = 0.001$) and MSC-2 ($^{*}P = 0.001$) groups compared with controls. NC: normal control; C: control (diabetes control without treatment); MSC-1: one administration of MSCs; MSC-2: two administrations of MSCs. (See color plate section.)

MSC-coated sutures may enhance wound healing and tissue remodeling through the release of different collagen types. The most abundant secreted collagen type was collagen alpha-1(I), which plays an important role in angiogenesis, invasion, and wound healing, as well as in the maintenance and function of the ECM [17].

Allogeneic MSCs derived from young healthy donors may have an advantage over autologous sources, where age and systemic disease can be significant factors [40].

Topical allogeneic MSC transplantation can accelerate the closure of experimental full-thickness cutaneous wounds and attenuate local inflammation. In a canine model, MSC-treated wounds showed decreased expression of the proinflammatory cytokines IL-2 and interferon-y and the wound-healing-related factors basic fibroblast growth factor and matrix metalloproteinase (MMP)-2 [18].

Hydrogels conform to the irregular shapes of wound beds and can be engineered to degrade at a rate that is compatible with the healing process [41].

The structure of the microenvironment can significantly alter MSC behavior and can be used to manipulate their cytokine secretion profile. VEGF-A and monocyte chemoattractant protein 1 both contribute significantly to the angiogenic response after wounding [19].

A number of clinical trials using MSCs for wound healing are registered on ClinicalTrials.gov (Table 59.2).

Challenges remain with MSC-based wound therapy. MSCs are heterogeneous and have a limited lifespan *in vitro* [42]. Bone marrow suppression has been noted in severe burns and trauma as a result of either sulfadiazine toxicity or sepsis, and may thus prevent adequate harvesting of autologous BM-MSCs [43]. Additionally it takes weeks to expand MSCs. The use of cryopreserved allogeneic MSCs as an "off the shelf" ready to use product may obviate these problems [38,44] (Figures 59.1 and 59.2).

Abundant experimental evidence has demonstrated the therapeutic potential of BM-MSCs for the regeneration and repair of damaged tissue in almost all of the major organs of the body, including the heart, brain, lung, liver, kidney, eye, and skin. BM-MSCs can also act as seed cells for the production of engineered tissues and as delivery vehicles for gene therapy [46].

## 59.4 The role of adipose-tissue-derived mesenchymal stromal cells in wound healing

One of the most abundant and accessible sources of adult stromal cells is adipose tissue. The subcutaneous fat in the lower abdomen site alone may provide as many as 100 to 1000 times more stromal cells than bone marrow [47]. A consistent cell surface marker profile has been determined for ADSCs [48] (see Table 59.3 and also Chapters 1 and 2).

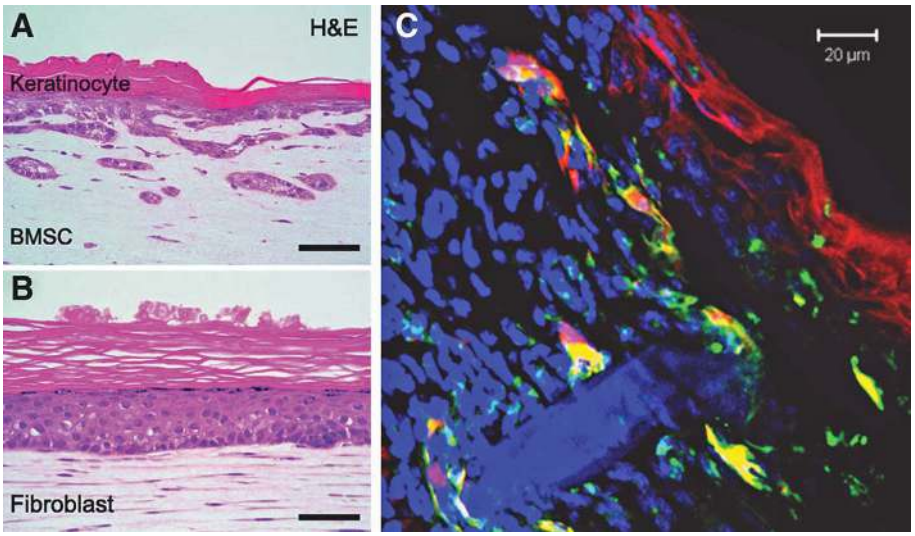



**Figure 59.2** BM-MSCs in cutaneous regeneration. (A, B) Keratinocytes loaded on a collagen gel containing BM-MSCs formed a rete ridge-like structure, whereas keratinocytes loaded on a collagen gel containing dermal fibroblasts did not (scale bars: 100 μm). (C). BM-MSCs (green) from green fluorescent protein-expressing BALB/c mice were injected around the excisional wound and applied to the wound bed in Matrigel. At day 7 some MSCs (yellow) expressed the keratinocyte marker cytokeratin (red) and formed structures similar to those observed in the study of Aoki *et al.* [45]. BMSC: BM-MSC; H&E: hematoxylin and eosin. (See color plate section.)

Wound healing experiments using the db/db mouse model have shown that application of autologous ADSCs containing atelocollagen matrix with a silicone membrane on to an open wound induced significant granulation tissue, epithelium, and capillary formations that accelerated wound healing [21]. (Cleavage of the antigenic telopeptide region from type I collagen yields atelocollagen, which is widely used as a scaffold for bone regeneration combined with cells and growth factors.) Furthermore, ADSC application in nude mice resulted in a thicker dermal portion of scars that that in the control group [22]. In a porcine full-layer wound model, perivascular adipose stromal cell localization suggested enhancement of blood supply by vascular neogenesis associated with higher levels of VEGF [23]. Using murine models, Lim and Yoo found that the ADSC-treated group showed significantly more rapid wound healing and had higher histological scores than those of the control group. ADSC-treated wounds had significantly higher capillary densities than the control group. The effects of ADSCs on wound healing may be due to stromal cell replacement by differentiation of ADSCs in the wound and the secretion of growth factors by the differentiated cells [24]. There is evidence that ADSCs facilitate tissue repair through paracrine mechanisms. CM from TGF-β1-treated ADSCs effectively promoted the proliferation and migration of human skin fibroblasts *in vitro* compared with nontreated control medium. In addition, the expression of MMP-1 was markedly increased by treatment with CM from TGF-β2-treated ADSCs. Expression of type I collagen in fibroblasts was slightly increased by treatment with CM from TGF-β1-treated ADSCs. The injection of CM from TGF-β1-treated ADSCs promoted the wound healing process in nude mice [25]. The effect of ADSC CM on the migration of endothelial cells, fibroblasts, and keratinocytes was different. Cell migration increased with increasing concentrations of ADSC CM and reached a maximum at 50% of ADSC CM. The optimal

**Table 59.3** Proteins of ADSCs.

| Molecule type | |
|---|---|
| Adhesion molecules | Positive for CD9, CD29, CD49d, ICAM-1, CD54, CD105, VCAM, CD106, ALCAM, CD166<br>Negative for CD11b, CD18, ICAM-3, CD50, NCAM, CD56, E-selectin, CD62 |
| Receptor molecules | CD44, CD71 |
| Surface enzymes | CD10, CD13, CD73 |
| Extracellular matrix proteins and glycoproteins | Type I and type III collagens, osteopontin, ostenectin (CD90), and MUC-18 (CD146) |
| Skeletal proteins | α-smooth muscle actin, vimentin |
| Hematopoietic cell marker proteins | Negative for CD14, CD31, CD45 |
| Complement regulatory proteins | CD55, complement protectin (CD59) |
| Histocompatibility antigens | Human leukocyte antigen-ABC |

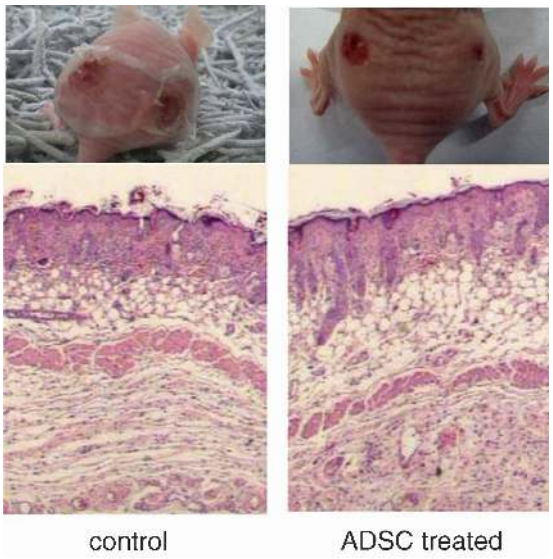



**Figure 59.3** Wound healing effect of ADSCs in nude mice. The wound size was reduced significantly in the ADSC-treated group (right side of the back) compared with the control group after 7 days. Reduced wound area was measured 7 days after surgery, and histological analysis of reconstructed skin using H&E staining was performed within 21 days of surgery. (See color plate section.)

timing for vascular endothelial cell migration and fibroblast migration was within 8 h and 24 h respectively [49]. Furthermore, hypoxia-conditioned media from ADSC cultures increased the rate of keratinocyte proliferation and migration. Antibody- and gel-based proteomic analysis of the ADSC CM showed upregulation of connective tissue growth factor, plasminogen activator inhibitor-1 (PAI-1/SERPINE1), hepatocyte growth factor (HGF), fibroblast growth factor (FGF)-1, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-6, VEGF, and TGF-β [50]. Additionally, ADSCs treated with the nuclear protein histone H1 showed upregulation of mitogen-activated protein kinase, ERK1/2, and sequential NF-κB translocation. This enhanced wound healing and increased IL-6 expression [51]. The use of ADSCs in wound treatment are shown in (Figure 59.3) [52].

Tissue engineering studies have indicated that ADSC sheets are a potentially viable matrix for healing full-thickness skin defects in a mouse model. Triple-layer ADSC sheet treatment significantly enhanced wound healing compared with the single-layer ADSC sheets at 7, 10, and 14 days. The density of blood vessels showed that ADSC sheet treatment slightly enhanced total vessel proliferation compared with controls [26].

A small but important finding has been made with the use of ADSCs in clinical trials. ADSCs were used for nasal skin necrosis. After antibiotics and debridement, ADSCs were injected intradermally and subcutaneously. The wound was completely re-epithelialized 10 days after injection without noticeable scar formation during 6-month follow-up [53]. ADSCs are thought to affect ischemic tissues by secreting angiogenic factors that stimulate angiogenesis, differentiation of ADSCs into vascular cells, and secretion of factors that enhance progenitor cell availability [54]. Many studies have indicated that stromal cells implement their therapeutic effects through paracrine mechanisms [55] (Table 59.4).

Studies using *ex vivo* expanded ADSCs have suggested cell populations are safe and effective in clinical use [56].

## 59.5 The role of mesenchymal stromal cells from placental tissues in wound healing

The origin of MSCs may have a significant impact on their therapeutic potential. Zhu *et al.* found that fetal but not maternal placental MSCs (pMSCs) expressed high levels of CD200 and HGF. (It is thought that CD200 may regulate myeloid cell activity and deliver an inhibitory signal to cells of the macrophage lineage.) $HGF^+$ and $CD200^+$ pMSCs had greater potential in promoting angiogenesis *in vitro* and increasing immunosuppressive function *in vivo* compared with $HGF^-$ and $CD200^-$ pMSCs [57].

**Table 59.4** Secretion of growth factors by ADSCs.

| Type of factor | Factor |
|---|---|
| Angiogenic | HGF, VEGF |
| Hematopoietic | Factor increasing monocytopoiesis-like tyrosine kinase-3 ligand, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IL-7, monocyte colony-stimulating factor |
| Proinflammatory | IL-6, IL-8, IL-11, leukemia inhibitory factor, TNF-α, basic FGF, TGF-α |

One study demonstrated that low oxygen tension regulated the fate of pMSCs from both early and late gestational tissues in response to insulin-like growth factor-1. However, phosphorylation of ERK1/2 and AKT was different between preterm and term pMSCs. Phosphorylated AKT, and not phosphorylated ERK1/2, was the major determinant of pMSC proliferation and octamer-4 levels [58].

The umbilical cord is a rich source of stromal cells. These cells also have low immunogenicity and thus could be applied as an allograft to accelerate healing of burns, thereby improving morbidity and mortality by reducing scarring [47]. Cord blood and different components of the cord, including Wharton's jelly (the gelatinous supporting matrix) and the outer lining, could also serve as a source of stromal cells [59,60].

Wharton's jelly MSCs (WJ-MSCs) and a decellularized amniotic membrane scaffold exhibited significantly better wound healing capabilities, reduced scar formation, improved hair growth, and improved biomechanical properties of regenerated skin compared with WJ-MSCs alone [27]. Human UC-MSCs improved wound healing in a rat model of severe burns. These data provide a foundation for the clinical application of human UC-MSCs in burns. Human UC-MSCs decreased the inflammatory cellular infiltrate and decreased the levels of the proinflammatory cytokines IL-1, IL-6, and TNF-α. They increased the levels of IL-10, TNF-α-induced protein-6, neovascularization, the levels of VEGF, and the ratio of collagen types I and III in a rat model of severe burns [28]. Moreover WJ-MSCs improved the healing response *in vivo* in a mouse model of dermal repair by triggering angiogenesis and providing a suitable matrix for wound repair without altering the inflammatory response. They were associated with stronger expression of VEGF-A, angiopoietin-1, and acidic FGF, which are all are directly involved in angiogenesis [61].

Human umbilical cord blood is also a rich source of hematopoietic stem cells, which can be used for hematopoietic stem cell transplantation, especially in children [62].

It has been reported that stromal cells isolated from umbilical cord blood can be differentiated into epithelial cells *in vitro* [63,64]. Expansion of such cells could be used for the treatment of large skin defects. A recent *in vivo* study indicated that MSCs derived from human umbilical cord blood improved skin wound healing [29].

One study optimized the methodology for isolation, culture expansion, characterization, differentiation and immunophenotyping of MSCs from equine umbilical cord blood. These MSCs were used for the repair of tendon and ligament injuries in race horses with good results [65].

Stromal cells isolated from the internal lining of umbilical cord are termed cord lining stem cells. Some of these cells are mesenchymal cells (cord lining mesenchymal cells). They express stromal cell markers and form colonies with typical stromal cell morphology in culture. Both *in vitro* and *in vivo* studies have shown their multipotency, including their potential to differentiate toward epidermal tissues [59].

MSCs from the fetal-derived amniotic membrane may also be useful clinically as they, too, are proangiogenic and antifibrotic, retard scar formation, and have low immunogenicity.

CM from amniotic-fluid-derived MSCs cultured under hypoxic conditions contains factors such as VEGF and TGF-β1. Amniotic membrane MSCs enable rapid vascularization and tissue restoration in wound healing. They enhance wound healing via activation of TGF-β/SMAD2 and phosphatidylinositide 3-kinase/AKT pathways [30].

Amniotic membrane MSCs can initiate endothelialization and angiogenesis *in vitro* and can form endothelial rings in Matrigel. Reverse transcriptase polymerase chain reaction showed expression of proangiogenic factors such as tyrosine kinase with immunoglobulin-like and epidermal growth factor-like domains (Tie2), angiopoietin-1, VEGF, VEGF receptor, von Willebrand factor, kinase insert domain receptor (a type III receptor tyrosine kinase also known as VEGF receptor 2), and factor increasing monocytopoiesis-like tyrosine kinase 4 [66]. pMSCs have been used to promote angiogenesis in order to accelerate wound healing in diabetic rats. They produced VEGF, HGF, basic FGF, TGF-β, and insulin-like growth factor-1 at bioactive levels [67].

Several clinical trials are ongoing (see Table 59.2) to evaluate these cells in the treatment of partial- and full-thickness burns and chronic diabetic wounds.

In general, UC-MSCs exhibited immunophenotypic and functional characteristics similar to those of BM-MSCs [20]. They represent a theoretical alternative to BM-MSCs for tissue repair and regeneration, although questions on aspects such as donor selection, culture conditions on clinical outcomes, and product consistency remain to be answered [68].

Fetal pMSCs cultured in serum-free medium have a tendency to modify methylation during *in vitro* expansion: 246 genes with differential DNA methylation of promoters were identified, along with significantly downregulated global DNA methyltransferase activity. Thus, epigenetic stability must be verified before fetal pMSCs are used clinical trials [69].

## 59.6 The role of mesenchymal stromal cells from dermal tissue in wound healing

Skin, the largest organ in the body, is composed of two layers: the epidermis, in which the cells form a protective barrier against external injury, and the dermis with its associated appendages, including hair follicles, sebaceous glands, and sweat glands, which provide nutritional support to the epidermis. Dermal stromal cells are the most likely candidates to be directly involved in the healing of deep skin wounds since dermal tissue is exposed at the wound bed margins. Epidermal-derived MSCs have been implicated in wound repair and regeneration. Recent advances in locating, identifying, and isolating a variety of adult stromal cell populations in skin and hair follicles have led to appreciation of

their potential clinical application. The ability of stromal cells to proliferate, as well as their multilineage differentiation potential, makes them candidates for skin regeneration strategies. MSCs residing in human adult dermis have not yet been described in detail. Vaculik *et al.* immunoselected CD271$^+$ (low-affinity nerve growth factor receptor expressed on MSCs) and stage-specific embryonic antigen-4$^+$ (SSEA-4$^+$ – expressed on early stem cells) cells from adherent dermal cells and investigated their mesenchymal differentiation capacity. Cells with increased *in vitro* adipogenic, osteogenic, and chondrogenic differentiation potential were enriched in the dermal CD271$^+$ population. The differentiation potential of dermal SSEA-4$^+$ cells, in contrast, appeared to be limited to cells that underwent adipogenesis. These results indicated that specific cell populations with variable mesenchymal differentiation potential can be isolated from human dermis, and three different subsets of dermal MSCs were identified [70]. Kroeze *et al.* showed that a population of MSCs resides in the dermis of human adult skin and that these dermal-derived stromal cells have a phenotypic and chemokine-mediated homing potential [71]. Dermal stromal cells display a cell surface phenotype similar to that of ADSCs: CD34$^+$, CD54$^+$, CD90$^+$, CD105$^+$, CD166$^+$ and CD45$^-$ and CD31$^-$. They also display a set of chemokine receptors that includes CCR3, CCR4, CCR6, CCR10, CXCR1, and CXCR2. CCL5/RANTES can induce migration of stromal cells [71]. Furthermore, wound healing requires the adjacent uninjured dermal mesenchymal cells for reconstituting the dermal fibroblast population, and 15–20% of the spindle-shaped dermal fibroblasts were bone marrow derived [72]. It will be important to determine the importance of cytokine gradients within the wound bed, for example, for epidermal-derived MSCs [71], and it will be important to determine the role of allogeneic stromal cells in the regulation of immune homeostasis within the wound [31].

## 59.7 The role of mesenchymal stromal cells from blood in wound healing

MSCs are widely distributed in body tissues and organs. Wound healing involves BM-MSCs for reconstitution of the dermal fibroblast population, since approximately a quarter of the spindle-shaped dermal fibroblasts present in the skin wound were bone marrow derived [72]. Further, Tian *et al.* suggested that human MSCs produced significant elevation of signals such as Wnt3a, VEGF, and platelet-derived growth factor receptor [14]. MSCs are important in recruiting existing endogenous tissue progenitor cells to enhance both normal and impaired wound healing [73].

Extracts of the bark of *Mallotus philippinensis*, a plant of the spurge family, was found to activate the mobilization of MSCs into the circulation and their homing to wound sites with improved wound healing in a diabetic mouse model [74]. HGF incorporated into collagen or fibrin gels led to enhanced and directed MSC migration *in vitro*. HGF-loaded biomaterials may be useful as *in vivo* wound dressings to recruit endogenous MSCs from tissue-specific niches to the wounded area [75]. Fibrin clot formation is associated with most wound healing processes and potentially guides the recruitment of human MSCs. Neuss *et al.* stated that the high intrinsic fibrinolytic capacity of human MSCs mediates their invasion into the fibrin clot in wounded tissue [13].

This novel approach may help to reduce the costly multistep procedures of cell isolation, *ex vivo* expansion culture, and transplantation normally used in MSC manufacturing.

## 59.8 Questions and challenges regarding mesenchymal stromal cell administration in wound healing

Further work in mouse models will improve our knowledge of the diversity and origin of dermal mesenchymal populations [76]. Whether MSCs facilitate wound repair through paracrine secretion to control inflammation, transdifferentiation, cell fusion with resident cells, or support for other stromal cells needs to be determined [44,77–80]. It is likely that a combination of these mechanisms will facilitate wound repair.

Future studies will need to determine the conditions for the production of MSC microvesicles and their efficiency in preclinical models compared with CM or MSCs themselves [81]. MSC-CM [82–84] and MSC-derived exosomes [85,86] are being increasingly explored (see Chapters 1, 20, and 38). The choice of autologous MSCs versus allogeneic MSCs needs to be determined [87].

## 59.9 Conclusions

MSCs have been shown to improve skin wound healing in multiple preclinical models and in a few clinical studies. The benefit of MSCs may be at least in part reproduced by the growth factors or immunomodulatory molecules that they produce. In this review we have highlighted important preclinical (Table 59.1) and clinical (Table 59.2) studies that have validated the significance of MSCs derived from bone marrow, placental tissue, dermal tissue, and blood in facilitating wound healing. The rapid progress in this field has indicated the emergence of important questions in skin biology that need to be addressed in parallel with the use of MSCs in the clinic.

## References

1 Atiyeh BS, Ioannovich J, Al-Amm CA, El-Musa KA. 2002. Management of acute and chronic open wounds: the importance of moist environment in optimal wound healing. *Curr Pharm Biotechnol* **3**:179–195.

2 Maxson S, Lopez EA, Yoo D, *et al*. 2012. Concise review: role of mesenchymal stem cells in wound repair. *Stem Cells Transl Med* **1**:142–149.

3 Corotchi MC, Popa MA, Remes A, *et al*. 2013. Isolation method and xeno-free culture conditions influence multipotent differentiation capacity of human Wharton's jelly derived mesenchymal stem cells. *Stem Cell Res Ther* **4**:81.

4 Portmann-Lanz CB, Schoeberlein A, Huber A, *et al*. 2006. Placental mesenchymal stem cells as potential autologous graft for pre-and perinatal neuroregeneration. *Am J Obstet Gynecol* **194**:64–73.

5 Musina RA, Belyavski AV, Tarusova OV, *et al*. 2008. Endometrial mesenchymal stem cells isolated from the menstrual blood. *Exp Biol Med* **145**:539–543.

6 Steigman SA, Fauza DO. 2007. Isolation of mesenchymal stem cells from amniotic fluid and placenta. *Curr Protoc Stem Cell Biol* **1**: Unit 1E.

7 Tirino V, Paino F, d'Aquino R, *et al*. 2011. Methods for the identification, characterization and banking of human DPSCs: current strategies and perspectives. *Stem Cell Rev* **7**:608–615.

8 Gebler A, Zabel O, Seliger B. 2012. The immunomodulatory capacity of mesenchymal stem cells. *Trends Mol Med* **18**:128–134.

9 Wu Y, Chen L, Scott PG, Tredget EE. 2007. Mesenchymal stem cells enhance wound healing through differentiation and angiogenesis. *Stem Cells* **25**:2648–2659.

10 Walter MN, Wright KT, Fuller HR, *et al*. 2010. Mesenchymal stem cell-conditioned medium accelerates skin wound healing: an *in vitro* study of fibroblast and keratinocyte scratch assays. *Exp Cell Res* **316**:1271–1281.

11 Stoff A, Rivera AA, Sanjib Banerjee N, *et al*. 2009. Promotion of incisional wound repair by human mesenchymal stem cell transplantation. *Exp Dermatol* **18**:362–369.

12 Chen L, Tredget EE, Liu C, Wu Y. 2009. Analysis of allogenicity of mesenchymal stem cells in engraftment and wound healing in mice. *PLoS ONE* **4**:e7119.

13 Neuss S, Schneider RK, Tietze L, *et al*. 2010. Secretion of fibrinolytic enzymes facilitates human mesenchymal stem cell invasion into fibrin clots. *Cells Tissues Organs* **191**:36–46.

14 Tian H, Lu Y, Shah SP, Hong S. 2011. 14*S*,21*R*-Dihydroxydocosahexaenoic acid remedies impaired healing and mesenchymal stem cell functions in diabetic wounds. *J Biol Chem* **286**:4443–4453.

15 Agay D, Scherthan H, Forcheron F, *et al*. 2010. Multipotent mesenchymal stem cell grafting to treat cutaneous radiation syndrome: development of a new minipig model. *Exp Hematol* **38**:945–956.

16 Xu J, Wu W, Zhang L, *et al*. 2012. The role of microRNA-146a in the pathogenesis of the diabetic wound-healing impairment. *Diabetes* **61**:2906–2912.

17 Casado JG, Blazquez R, Jorge I, *et al*. 2014. Mesenchymal stem cell-coated sutures enhance collagen depositions in sutured tissues. *Wound Rep Reg* **22**:256–264.

18 Kim J-W, Lee J-H, Lyoo Y-S, *et al*. 2013. The effects of topical mesenchymal stem cell transplantation in canine experimental cutaneous wounds. *Vet Dermatol* **24**:242–253.

19 Rustad KC, Wong VW, Sorkin M, *et al*. 2012. Enhancement of mesenchymal stem cell angiogenic capacity and stemness by a biomimetic hydrogel scaffold. *Biomaterials* **33**(1):80–90.

20 François S, Mouiseddine, Mathiu N, *et al*. Human mesenchymal stem cells favour healing of the cutaneous radiation syndrome in a xenogenic transplant model. *Ann Hematol* 2007;**86**(1):1–8.

21 Nambu M, Kishimoto S, Nakamura S, *et al*. 2009. Accelerated wound healing in healing-impaired db/db mice by autologous adipose tissue-derived stromal cells combined with atelocollagen matrix. *Ann Plast Surg* **62**(3):317–321.

22 Lee SH, Lee JH, Cho KH. 2011. Effects of human adipose-derived stem cells on cutaneous wound healing in nude mice. *Ann Dermatol* **23**(2):150–155.

23 Blanton MW, Hadad I, Johnstone BH, *et al*. 2009. Adipose stromal cells and platelet-rich plasma therapies synergistically increase revascularization during wound healing. *Plast Reconstr Surg* **123**:56S–64S.

24 Lim JS, Yoo G. 2010. Effects of adipose-derived stromal cells and of their extract on wound healing in a mouse model. *J Korean Med Sci* **25**(5):746–751.

25 Cho J.-W., Kang M.-C., Lee K.-S. 2010. TGF-β1-treated ADSCs-CM promotes expression of type I collagen and MMP-1, migration of human skin fibroblasts, and wound healing *in vitro* and *in vivo*. *Int J Mol Med* **26**:901–906.

26 Lin Y-C, Grahovac T, Oh SJ, *et al*. 2013. Evaluation of a multi-layer adipose-derived stem cell sheet in a full-thickness wound healing model. *Acta Biomater* **9**:5243–5250.

27 Sabapathy V, Sundaram B, Mankuzhy P, *et al*. 2014. Wharton's jelly mesenchymal stem cells plasticity augments scar-free skin wound healing with hair growth. *PLoS ONE* **9**(4):e93726.

28 Liu L, Yu Y, Hou Y, *et al*. 2014. Human umbilical cord mesenchymal stem cells transplantation promotes cutaneous wound healing of severe burned rats. *PLoS ONE* **9**(2):e88348.

29 Luo G, Cheng W, He W, *et al*. 2010. Promotion of cutaneous wound healing by local application of mesenchymal stem cells derived from human umbilical cord blood. *Wound Repair Regen* **18**:506–513.

30 Jun EK, Zhang Q, Yoon BS, *et al*. 2014. Hypoxic conditioned medium from human amniotic fluid-derived mesenchymal stem cells accelerates skin wound healing through TGF-α/SMAD2 and PI3K/Akt pathways. *Int J Mol Sci* **15**:605–628.

31 Pfisterer K, Lipnik KM, Hofer E, Elbe-Bürger A. 2015. $CD90^+$ human dermal stromal cells are potent inducers of $FoxP3^+$ regulatory T cells. *J Invest Dermatol* **135**(1):130–141.

32 Katebi M, Soleimani M, Cronstein BN. 2009. Adenosine A2A receptors play an active role in mouse bone marrow-derived mesenchymal stem cell development. *J. Leukoc Biol* **85**:438–444.

33 Busletta C, Novo E, Valfrè Di Bonzo L, *et al*. 2011. Dissection of the biphasic nature of hypoxia-induced motogenic action in bone marrow-derived human mesenchymal stem cells. *Stem Cells* **29**:952–963.

34 Ulivi V, Tasso R, Cancedda R, Descalzi F. 2014. Mesenchymal stem cell paracrine activity is modulated by platelet lysate: induction of an inflammatory response and secretion of factors maintaining macrophages in a proinflammatory phenotype. *Stem Cells Dev* **23**(16):1858–1869.

35 Alfaro MP, Vincent A, Saraswati S, *et al*. 2010. sFRP2 suppression of bone morphogenic protein (BMP) and Wnt signaling mediates mesenchymal stem cell (MSC) self-renewal promoting engraftment and myocardial repair. *J Biol Chem* **285**:35645–35653.

36 Zhang B, Wang M, Gong A, *et al*. 2015. HucMSC-exosome mediated-Wnt4 signaling is required for cutaneous wound healing. *Stem Cells* **33**:2158–2168.

37 McGrail DJ, McAndrews KM, Dawson MR. 2013. Biomechanical analysis predicts decreased human mesenchymal stem cell function before molecular differences. *Exp Cell Res* **319**:684–696.

38 Tschumperlin DJ, Liu F, Tager AM. 2013. Biomechanical regulation of mesenchymal cell function. *Curr Opin Rheumatol* **25**:92–100.

39 Kuo Y-R, Wang C-T, Cheng J-T, *et al.* 2011. Bone marrow-derived mesenchymal stem cells enhanced diabetic wound healing through recruitment of tissue regeneration in a rat model of streptozotocin-induced diabetes. *Plast Reconstr Surg* **128**:872–880.

40 Badiavas AR, Badiavas EV. 2011. Potential benefits of allogeneic bone marrow mesenchymal stem cells for wound healing. *Expert Opin Biol Ther* **11**(11):1447–1454.

41 Salinas CN, Anseth KS. 2009. Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. *J Dent Res* **88**:681–692.

42 Mohanty ST, Cairney CJ, Chantry AD, *et al.* 2012. A small molecule modulator of prion protein increases human mesenchymal stem cell lifespan, *ex vivo* expansion, and engraftment to bone marrow in NOD/SCID mice. *Stem Cells* **30**:1134–1143.

43 Butler KL, Goverman J, Ma H, *et al.* 2010. Stem cells and burns: review and therapeutic implications. *J Burn Care Res* **31**:874–881.

44 Wu Y, Zhao RCH, Tredget EE. 2010. Concise review: bone marrow-derived stem/progenitor cells in cutaneous repair and regeneration. *Stem Cells* **28**:905–915.

45 Aoki S, Toda S, Ando T, Sugihara H. Bone marrow stromal cells, preadipocytes, and dermal fibroblasts promote epidermal regeneration in their distinctive fashions. *Mol Biol Cell* 2004;**15**:4647–4657.

46 Chen L, Xu Y, Zhao J, *et al.* 2014. Conditioned medium from hypoxic bone marrow-derived mesenchymal stem cells enhances wound healing in mice. *PLoS ONE* **9**(4):e96161.

47 Aust L, Devlin B, Foster SJ, *et al.* 2004. Yield of human adipose derived adult stem cells from liposuction aspirates. *Cytotherapy* **6**:7–14.

48 Gronthos S, Franklin DM, Leddy HA, *et al.* 2001. Surface protein characterization of human adipose tissue-derived stromal cells. *J Cell Physiol* **189**:54–63.

49 Hu L, Zhao J, Liu J, *et al.* 2013. Effects of adipose stem cell-conditioned medium on the migration of vascular endothelial cells, fibroblasts and keratinocytes. *Exp Ther Med* **5**(3):701–706.

50 Moon KM, Park YH, Lee JS, *et al.* 2012. The effect of secretory factors of adipose-derived stem cells on human keratinocytes. *Int J Mol Sci* **13**:1239–1257.

51 Hsu L-W, Goto S, Nakano T, *et al.* 2012. The effect of exogenous histone H1 on rat adipose-derived stem cell proliferation, migration, and osteogenic differentiation *in vitro*. *J Cell Physiol* **227**:3417–3425.

52 Kim W-S, Park B-S, Sung J-H, *et al.* 2007. Wound healing effect of adipose-derived stem cells: a critical role of secretory factors on human dermal fibroblasts. *J Dermatol Sci* **48**:15–24.

53 Sung HM, Suh IS, Lee HB, *et al.* 2012. Case reports of adipose-derived stem cell therapy for nasal skin necrosis after filler injection. *Arch Plast Surg* **39**(1):51–54.

54 Hong SJ, Traktuev DO, March KL. 2010. Therapeutic potential of adipose-derived stem cells in vascular growth and tissue repair. *Curr Opin Organ Transplant* **15**:86–91.

55 Kilroy GE, Foster SJ, Wu X, *et al.* 2007. Cytokine profile of human adipose-derived stem cells: expression of angiogenic, hematopoietic, and pro-inflammatory factors. *J Cell Physiol* **212**(3):702–709.

56 Kolle SF, Fischer-Nielsen A, Mathiasen AB, *et al.* 2013. Enrichment of autologous fat grafts with *ex-vivo* expanded adipose tissue-derived stem cells for graft survival: a randomized placebo-controlled trial. *Lancet* **382**:1113–1120.

57 Zhu Y, Yang Y, Zhang Y, *et al.* 2014. Placental mesenchymal stem cells of fetal and maternal origins demonstrate different therapeutic potentials. *Stem Cell Res Ther* **5**(2):48.

58 Youssef A, Iosef C, Han VK. 2014. Low-oxygen tension and IGF-I promote proliferation and multipotency of placental mesenchymal stem cells (PMSCs) from different gestations via distinct signaling pathways. *Endocrinology* **155**(4):1386–1397.

59 Huang L, Wong YP, Gu H, *et al.* 2011. Stem cell-like properties of human umbilical cord lining epithelial cells and the potential for epidermal reconstitution. *Cytotherapy* **13**:145–155.

60 Can A, Karahuseyinoglu S. 2007. Concise review: human umbilical cord stroma with regard to the source of fetus-derived stem cells. *Stem Cells* **25**:2886–2895.

61 Edwards SS, Zavala G, Prieto CP, *et al.* 2014. Functional analysis reveals angiogenic potential of human mesenchymal stem cells from Wharton's jelly in dermal regeneration. *Angiogenesis* **17**(4): 851–866.

62 Rao M, Ahrlund-Richter L, Kaufman DS. 2012. Concise review: cord blood banking, transplantation and induced pluripotent stem cell: success and opportunities. *Stem Cells* **30**:55–60.

63 Kamolz LP, Kolbus A, Wick N, *et al.* 2006. Cultured human epithelium: human umbilical cord blood stem cells differentiate into keratinocytes under *in vitro* conditions. *Burns* **32**:16–19.

64 Mortier L, Delesalle F, Formstecher P, *et al.* 2010. Human umbilical cord blood cells form epidermis in the skin equivalent model. *Exp Dermatol* **19**:929–930.

65 Mohanty N, Gulati BR, Kumar R, *et al.* 2014. Phenotypical and functional characteristics of mesenchymal stem cells derived from equine umbilical cord blood. *Cytotechnology*. doi: 10.1007/s10616-014-9831-z

66 Warrier S, Haridas N, Bhonde R. 2012. Inherent propensity of amnion-derived mesenchymal stem cells towards endothelial lineage: vascularization from an avascular tissue. *Placenta* **33**:850–858.

67 Kong P, Xie X, Li F, *et al.* 2013. Placenta mesenchymal stem cell accelerates wound healing by enhancing angiogenesis in diabetic Goto-Kakizaki (GK) rats. *Biochem Biophys Res Commun* **438**:410–419.

68 Yang S, Huang S, Feng C, Fu X. 2012. Umbilical cord-derived mesenchymal stem cells: strategies, challenges, and potential for cutaneous regeneration. *Front Med* **6**:41–47.

69 Zhu Y, Song X, Wang J, *et al.* 2015. Placental mesenchymal stem cells of fetal origin deposit epigenetic alterations during long-term culture under serum-free condition. *Expert Opin Biol Ther* **15**(2):163–180.

70 Vaculik C, Schuster C, Bauer W, *et al.* 2012. Human dermis harbors distinct mesenchymal stromal cell subsets. *J Invest Dermatol* **132**(3 Pt 1):563–574.

71 Kroeze KL, Jurgens WJ, Doulabi BZ, *et al.* 2009. Chemokine-mediated migration of skin-derived stem cells: predominant role for CCL5/RANTES. *J Invest Dermatol* **129**:1569–1581.

72 Fathke C, Wilson L, Hutter J, *et al.* 2004. Contribution of bone marrow-derived cells to skin: collagen deposition and wound repair. *Stem Cells* **22**(5):812–822.

73 Shin L, Peterson DA. 2013. Human mesenchymal stem cell grafts enhance normal and impaired wound healing by recruiting existing endogenous tissue stem/progenitor cells. *Stem Cells Transl Med* **2**:33–42.

74 Furumoto T, Ozawa N, Inami Y, *et al.* 2014. *Mallotus philippinensis* bark extracts promote preferential migration of mesenchymal stem

cells and improve wound healing in mice. *Phytomedicine* **21**:247–253.

75 Van de Kamp J, Jahnen-Dechent W, Rath B, *et al*. 2013. Hepatocyte growth factor-loaded biomaterials for mesenchymal stem cell recruitment. *Stem Cells Int* **2013**:892065.

76 Enshell-Seijffers D, Lindon C, Kashiwagi M, Morgan BA. 2010. β-catenin activity in the dermal papilla regulates morphogenesis and regeneration of hair. *Dev Cell* **18**:633–642.

77 Khosrotehrani K. 2013. Mesenchymal stem cell therapy in skin: why and what for? *Exp Dermatol* **22**:307–310.

78 Sasaki M, Abe R, Fujita Y, *et al*. 2008. Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *J Immunol* **180**:2581–2587.

79 Spees JL, Olson SD, Ylostalo J, *et al*. 2003. Differentiation, cell fusion, and nuclear fusion during *ex vivo* repair of epithelium by human adult stem cells from bone marrow stroma. *Proc Natl Acad Sci U S A* **100**:2397–2402.

80 Wong VW, Crawford JD. 2013. Vasculogenic cytokines in wound healing. *Biomed Res Int* **2013**:190486.

81 Van Koppen A, Joles JA, van Balkom BW, *et al*. 2012. Human embryonic mesenchymal stem cell-derived conditioned medium rescues kidney function in rats with established chronic kidney disease. *PLoS ONE* **7**:e38746.

82 Heo SC, Jeon ES, Lee IH, *et al*. 2011. Tumor necrosis factor-α-activated human adipose tissue-derived mesenchymal stem cells accelerate cutaneous wound healing through paracrine mechanisms. *J Invest Dermatol* **131**:1559–1567.

83 Yew TL, Hung YT, Li HY, *et al*. 2011. Enhancement of wound healing by human multipotent stromal cell conditioned medium: the paracrine factors and p38 MAPK activation. *Cell Transplant* **20**:693–706.

84 Chen L, Tredget EE, Wu PYG, Wu Y. 2008. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* **3**:e1886.

85 Lai RC, Arslan F, Lee MM, *et al*. 2010. Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* **4**:214–222.

86 Lai RC, Chen TS, Lim SK. 2011. Mesenchymal stem cell exosome: a novel stem cell-based therapy for cardiovascular disease. *Regen Med* **6**:481–492.

87 Badiavas AR, Badiavas EV. 2011. Potential benefits of allogeneic bone marrow mesenchymal stem cells for wound healing. *Expert Opin Biol Ther* **11**(11):1447–1454.

SECTION VII

# Mesenchymal stromal cells as delivery vehicles for therapeutic agents

CHAPTER 60

# The role of mesenchymal stromal cells in human brain tumors

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## 60.1 Introduction

Glioblastoma multiforme (GBM), which is classified as World Health Organization grade IV astrocytoma, is the most common primary malignant brain tumor in adults. Despite surgery and radiation along with concurrent and adjuvant chemotherapy, patients with glioblastoma survive on average for only 14.6 months [1]. A radiological image of a GBM is shown in Figure 60.1.

This poor outcome is due at least in part to (1) the presence of glioma-initiating cells (GICs), which drive glioma formation and are intrinsically resistant to most therapies; (2) the complex microenvironment in which GICs reside and which enhances therapeutic resistance; and (3) the blood–brain and/or blood–tumor barriers (BBB/BTB), which limit the movement of most therapeutic agents into tumor-infiltrated brain tissue.

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In this context, recent studies have suggested that mesenchymal stromal cells (MSCs) may be important in both the therapy and biology of GBMs (Figure 60.2 and Table 60.1).

With regard to cell therapy, accumulating evidence has shown that MSCs have the capacity to migrate toward GBMs after intratumor injection, can overcome the BBB/BTB and home to GBMs after intravascular injection, and can be engineered to carry antitumor agents to gliomas. Therefore, exogenously engineered MSCs may be used as novel cellular vehicles to deliver various antiglioma agents for the therapy of GBMs [7–9]. With regard to the biology of gliomas, it has been suggested that mesenchymal stem/stromal cells, or at least cells that resemble them, may contribute to the complex microenvironment and stroma of GBMs. Thus, endogenous MSC-like cells may influence the biological behavior of GICs and may enhance the aggressive behavior and therapeutic resistance of GBMs. This suggests that these endogenous glioma-associated MSC-like cells may themselves represent a novel therapeutic target for GBM.

In this chapter we review recent studies describing the application of exogenous MSCs as cellular vehicles to deliver various

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**Figure 60.1** A GBM shown by magnetic resonance imaging of the brain. GBM usually exhibits a "ring" or "ring-like" zone of contrast enhancement around a dark central area of necrosis. *Source*: Armed Forces Institute of Pathology.

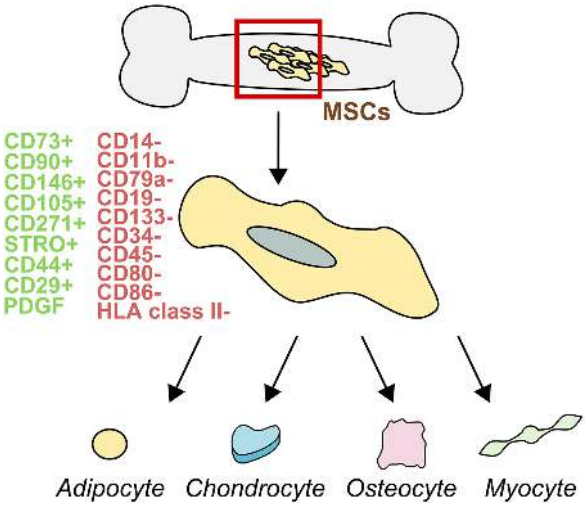



**Figure 60.2** Mesenchymal stem cells express a variety of surface markers and can differentiate into various cell types. (See color plate section.)

antiglioma therapies to GBMs. We also explore the tropism driving MSCs to home to brain tumors, and the various cargoes that have been engineered to be delivered by MSCs. We then briefly explore the controversial role of endogenous MSCs in gliomas and the possible influence of glioma-associated MSCs on the growth of gliomas.

## 60.2 Mesenchymal stromal cells in the therapy of human gliomas

Although hematopoietic stem cells have been utilized for decades in cancer therapy to replenish ablated bone marrow following aggressive chemotherapy or chemoradiotherapy as part of autologous, allogeneic or syngeneic hematopoietic stem cell (HSC) transplants, more recently the potential application of cells in cancer therapy has expanded to their use as novel cellular vehicles for delivering anti-tumor therapies to solid tumors including brain tumors.

## 60.3 Cellular therapy for gliomas

Neural stem cells (NSCs) were the first stem cells to be studied as potential cellular vehicles to deliver antitumor agents to brain tumors, beginning with the seminal publication of Aboody *et al.* in 2000 describing the use of NSCs in the treatment of gliomas [10]. NSCs are multipotent tissue-resident lineage-restricted stem cells that give rise to the cells that form the nervous system and that reside in the developing and adult central nervous system of mammals in the subependymal zone lining the lateral

**Table 60.1** Features of MSCs.

| Feature | Notes | References |
|---|---|---|
| Location | Bone marrow<br>Umbilical cord blood<br>Adipose tissue<br>All vascularized tissues | [2–4] |
| Cell fate | Adipocytes<br>Osteocytes<br>Chondrocytes<br>Mesenchymal elements within vascular niche | [2,5] |
| Expression markers | Positive: CD105, CD73, CD90, CD44, CD146, CD29, PDGF<br>Negative: CD45, CD34, CD14, CD11b, CD79alpha, CD19, HLA-DR, CD133 | [6] |
| Characteristics | Adhere to culture plastic and form colony-forming unit–fibroblasts *in vitro* and bone ossicles *in vivo* | [2] |

HLA: human leukocyte antigen; PDGF: platelet-derived growth factor.

ventricles and the dentate gyrus of the hippocampus [11–14]. NSCs can be cultured and expanded *in vitro* as floating cellular aggregates known as neurospheres. Beyond their capacity for self-renewal and differentiation, the two key properties of all true stem cells, NSCs possess an intrinsic capacity for extensive migration within the brain [15]. Investigators therefore reasoned that these migratory properties might allow NSCs to track down widely dispersed tumor cells that reside outside the main tumor mass and which are responsible for recurrence and treatment failure. Based on this hypothesis, Aboody *et al.* first described the application of an immortalized mouse NSC cell line, C17.2, to deliver a therapeutic gene to treat human glioma in a mouse model system [10]. They showed that these C17.2 NSCs could distribute themselves throughout the tumor mass and could home to infiltrating single tumor cells outside the main tumor mass. Equally importantly, they showed that NSCs could be engineered to carry the therapeutic transgene cytosine deaminase, an enzyme that converts the prodrug 5-fluorocytosine to 5-fluorouracil. This publication set the field of cell-based therapies for gliomas in motion [7–10,16–24].

## 60.4 The advantages of mesenchymal stromal cells in clinical use

Despite the promise of NSCs in brain tumor therapy, their acquisition for clinical use in human patients is complex and raises ethical concerns. In early preclinical studies NSCs were only obtained from mice because the isolation of human NSCs required human embryos or open surgical biopsy of the periventricular zone. Although human NSCs derived from aborted fetuses were eventually developed, and one human NSC line, HB1.F3, is currently in a clinical trial, the ethical issues and the complexity of acquiring human NSCs drove many investigators to search for more easily acquired stem cells that could equally home to brain tumors. In this context, investigators turned to the bone marrow, which was known to be a rich source of stem cells. Bone marrow is a particularly good source of stem cells for patients with brain tumors because most brain tumors do not metastasize to the bone marrow, so tumor contamination is not likely. Compared with NSCs, bone marrow stem cells are more attractive because (1) they are easily acquired from patients by aspiration of the iliac crest, (2) patients can act as their own donors, making autologous transplantation possible and obviating immune-mediated rejection, and (3) no ethical issues arise from their use.

There are two types of adult stem cell in human bone marrow. The first, as indicated earlier, is the HSC that has been used in mainstream clinical medicine since 1969 in HSC transplantation for a wide range of diseases, particularly malignancies of the hematopoietic system. HSCs are true stem cells, as shown by their twin abilities of self-renewal to daughter stem cells and to committed differentiation to a specific lineage – in this case the hematopoietic lineage – and thus provide all the functional cells of the hematopoietic and immune systems (see Chapter 57).

The other adult stem cell that occurs in the bone marrow is the mesenchymal stem cell; again, this cell can self-renew as well as differentiate down a specific lineage – in this case the mesodermal lineage – to produce the tissues of the skeleton. It can form bone *in vivo*, and *in vitro* can differentiate to osteogenic, adipogenic, and chondrogenic lineages if induced with appropriate chemical stimuli. For this reason it has been termed the stem cell of the skeletal system.

However, true mesenchymal stem cells are rare cells with a frequency in bone marrow estimated to be between 1 in $10^4$ and 1 in $10^5$ cells. It is thus difficult to obtain enough them to use clinically. Instead, their progeny, mesenchymal *stromal* cells, are used since they can be expanded *ex vivo* in enormous numbers. These are not true stem cells but they can regenerate bone and tendon tissue *in vivo* as well as differentiate down the osteogenic, adipogenic, and chondrogenic lineages if subjected to appropriate chemical stimuli *in vitro* (see Chapters 8, 9, 10, 11, 12, 13, 14, and 15).

They are also known as mesenchymal progenitor cells and are a transit amplifying population. As well as their use in clinical trials to regenerate or repair musculoskeletal tissues, they have another set of unique functions. They secrete a large number of proteins that have immunomodulatory, antiapoptotic, antifibrotic, and anti-inflammatory functions (see Chapters 33 and 34). Their use in many diseases outside the musculoskeletal system is based on these properties (see Chapters 18, 19, 20, 21, 37, and 38).

They have been called drug factories and mini-pharmacies. They form part of the endothelial wall and are thus a form of pericyte. They are thus present in all vascularized organs and tissues. They will therefore be close to any injury that occurs in a vascularized tissue or organ, and can presumably drop into, or migrate to, the injury site where it is likely that they will take cues from the local injured microenvironment and behave appropriately, be it to regenerate injured musculoskeletal tissues or to secrete appropriate proteins or both in contributing to healing of the injury. The abbreviation "MSC" in this chapter refers to mesenchymal *stromal* cells. Their characteristics are summarized in Table 60.1 and a comparison between NSCs and MSCs is shown in Table 60.2.

## 60.5 The rationale for using mesenchymal stromal cells in glioma therapy

Although MSCs had displayed the ability to home to sites of tissue injury such as myocardial infarction [31], muscular dystrophy [32], stroke [33], and Parkinsonism [34], it was not until 2002 that Studeny *et al.* showed that bone-marrow-derived MSCs (BM-MSCs) had a tropism for solid tumors and could therefore be applied in oncological disorders [35]. Reasoning that tumors act in many ways like "wounds that do not heal" [36],

**Table 60.2** The advantages and disadvantages of using NSCs and MSCs.

| Cell type | Advantages | Disadvantages | References |
|---|---|---|---|
| NSC | Primed for neural cell fate<br>Extensive migration within the brain | Difficult to obtain and expand in culture<br>Possibility of immune attack if allogeneic cells<br>Ethical concerns regarding cell acquisition | [10,25] |
| MSC | Easy to obtain with standard isolation techniques<br>No ethical concerns<br>May have better homing after vascular delivery<br>Easy to engineer | May promote tumor growth | [26–30] |

Studeny *et al.* hypothesized that MSCs may show a similar tropism to tumors as they do toward injured tissues. Indeed, in their seminal paper they showed that BM-MSCs could home to the site of human melanoma and deliver the antitumor cytokine interferon (IFN)-β [35]. Since then MSCs have been shown to display tropism in animal models of a variety of solid tumors, including sarcoma [37,38], lung [39], colon [40], ovary [41], pancreas [42], kidney [43] and breast cancers [44]. However, early on it was unclear whether MSCs would show a similar affinity for gliomas, which harbor an astrocytic rather than fibroblastic stroma and are protected by the BBB/BTB.

This concern regarding gliomas was addressed by two key reports. In the first, Nakamura *et al.* described the ability of rat MSCs to migrate toward gliomas in a rat syngeneic glioma model [17]. Two weeks after direct intracranial injection of green-fluorescent-protein-labeled rat MSCs into rat gliomas, the MSCs were shown to migrate to the periphery of the tumor. In specimens injected with MSCs into the hemisphere contralateral to the tumor, staining showed MSCs crossing the corpus callosum and migrating toward the tumor. These investigators also showed that rat MSCs could be engineered to deliver interleukin (IL)-2 as an antiglioma agent. This study was the first to document the ability of MSCs to migrate toward gliomas following intratumor or intracerebral injection in animal models.

In the second report, Nakamizo *et al.* showed for the first time that human MSCs not only possess the ability to migrate towards human gliomas after intracranial injection, but they also were capable of homing to gliomas after intravascular injection [7]. In these experiments MSCs were obtained from the bone marrow of normal human donors, cultured and labeled with a fluorescent dye, and injected into the carotid artery of nude mice harboring intracranial human glioma xenografts. Analyses of the brain specimens revealed fluorescence-labeled MSCs exclusively within the tumor, while MSCs were not seen in the normal brain parenchyma. The selectivity of the tropism of MSCs for human gliomas was further supported in similar experiments in which human MSCs were injected into the carotid artery contralateral to the tumor. Once again, MSCs localized exclusively to the tumor mass. Interestingly, carotid artery injection of labeled human fibroblasts did not result in engraftment, indicating that MSCs possess a unique capacity to home to human gliomas. Equally importantly, these workers again showed that MSCs could be engineered to carry the antitumor cytokine IFN-β, and that intravascular delivery of these engineered MSCs extended the survival of mice harboring intracranial gliomas. This finding was important from the perspective of clinical application, as it would allow for the less invasive intra-arterial delivery of MSCs and would allow repeat treatments, which is not possible with direct intracranial injection because it necessitates invasive surgery (Figure 60.3).

Many of these initial reports relied heavily on experimental models using "professional" glioma cell lines in rats or immunocompromised mice, which raised concerns about the clinical applicability of MSCs in glioma therapy. To address these concerns Doucette *et al.* took advantage of the RCAS/Ntv-a mouse glioma model in order to determine the extent to which MSCs isolated from Ntv-a mice are capable of localizing to endogenous high-grade gliomas [45]. The RCAS/Ntv-a mouse model employs somatic transfer of a gene of interest to glial progenitor cells in an immunocompetent mouse [46,47]. Unlike syngeneic grafting or xenograft mouse models, gliomas generated in the RCAS/Ntv-a mice form spontaneously in native cells after transfer of oncogenes and may more accurately mimic the infiltrative nature of gliomas than xenograft models. Building on

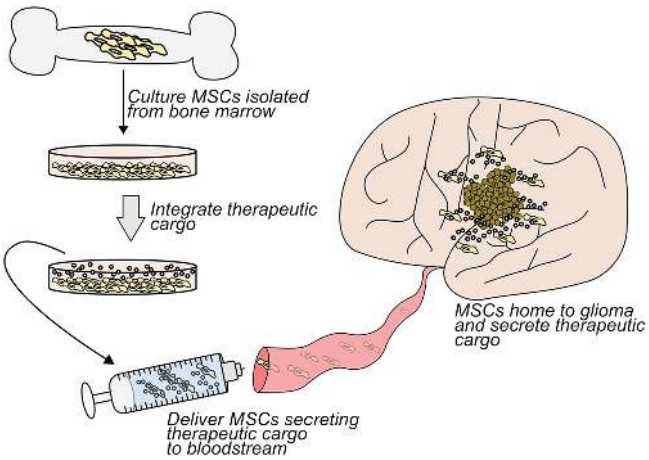



**Figure 60.3** MSCs injection technique. MSCs can be isolated from bone marrow, expanded in culture, and then implanted intravascularly. These MSCs then home to glioma, releasing antiglioma cargo. (See color plate section.)

the initial work of the Holland laboratory [46], the Zhang laboratory found that somatic transfer of PDGF subunit B along with insulin-like growth factor-binding protein 2 led to formation of endogenous murine gliomas with histological architecture remarkably similar to that of human GBMs with diffuse infiltration, microvascular proliferation, and pseudopallisading necrosis [48]. Doucette *et al*. utilized this model to study whether MSCs home to endogenous GBMs. MSCs from the bone marrow of Ntv-a mice were labeled with the dye SP-DiI or with luciferase and injected into the carotid artery of 4- to 6-week-old mice. Results showed that mice harboring infiltrating tumors exhibited luciferase activity. Fluorescent microscopy of brains from mice injected with SP-DiI-labeled MSCs revealed homing of MSCs to the tumor site but not in the surrounding brain parenchyma [45]. Demonstration of this tropism to glioma in the native brain microenvironment and in mice with an intact immune system provided further evidence for the applicability of MSCs for glioma therapy in the clinical setting.

Of equal interest is evidence that has suggested that human GBMs are driven by a population of cells with stem-like properties called GICs and glioma stem cells. Unlike commercially available glioma cell lines, including U87 and D54, GICs faithfully recapitulate human glioma characteristics and are considered the gold standard model for studying human gliomas *in vivo*. GICs represent the rare fraction of cells with stem cell properties that maintain the growth of human gliomas, are resistant to most therapies, and may cause persistent recurrences. Originally described by Singh *et al*., GICs can be isolated from surgical glioma specimens, grown as spheroids *in vitro*, and express variable levels of CD133 [49,50]. GICs isolated from GBM specimens grow intracranial gliomas with varying degrees of invasiveness, necrosis, and microvascular proliferation, recapitulating patients' GBMs. To determine whether MSCs may be able to deliver antiglioma agents to GICs, Shinojima *et al*. studied the capacity of human MSCs to home to xenografts derived from GICs [51]. They demonstrated that human BM-MSCs homed to xenografts derived from these GICs, and that MSCs could deliver therapeutic agents such as oncolytic adenoviruses to these tumors with significant therapeutic effects. These studies, including those of Doucette *et al*., confirmed that MSCs are highly efficient at delivering antiglioma cargoes to human gliomas in mouse models that faithfully mimic human gliomas [45,51].

Numerous publications in various experimental model systems have now verified that MSCs have a tropism for human gliomas and are highly effective at delivering a variety of cargoes to gliomas (Figure 60.3). This was originally conceived as an autologous transplant strategy in which MSCs derived from the patient would be modified and then delivered back to the same patient. However, it has now been shown that MSCs are relatively nonimmunogenic, as well as being suppressive of cellular immunity. Therefore, it has been suggested that MSCs derived from normal donors unrelated to, and major-histocompatibility-complex-unmatched with, the MSC recipient could be used in an allogeneic transplantation strategy. This approach is already being used in the majority of clinical trials involving MSCs (see ClinicalTrials.gov). This simplifies the use of MSCs, since culturing autologous MSCs takes at least 3–4 weeks, thus delaying their administration. Allotransplantation obviates such a delay since donor-derived MSCs can be manufactured in bulk and cryopreserved until needed in an acute or chronic medical setting. Future clinical trials should test the safety and feasibility of both of these approaches.

## 60.6 Mechanisms underlying mesenchymal stromal cell tropism for gliomas

It is generally agreed that the ability of MSCs to home to gliomas is mediated by factors secreted by the tumor cells that interact with surface receptors on the MSCs. MSCs respond to signals from injured tissues or organs and can then repopulate or repair these tissues. Because the microenvironment of GBMs (like that of other tumors) contains MSC-tropic factors similar to those found in injured tissue, related studies suggest that tumor-derived cytokines, chemokines, and growth factors may mediate the attraction of MSCs to brain tumors.

Transwell migration assays have been used to investigate the ability of cells to migrate toward specific growth factors *in vitro*. A study by Nakamizo *et al*. illustrated that chemokines and growth factors, including epidermal growth factor (EGF), stromal-cell-derived factor 1 – also known as C–X–C motif chemokine receptor (CXCR)12 – and PDGF-BB, promote MSC migration toward gliomas [7]. These studies also demonstrated that vascular endothelial growth factor and fibroblast growth factor may play a limited role in MSC migration. Similarly, Birnbaum *et al*. [52] demonstrated that the tropism of MSCs to glioma is mediated by angiogenic cytokines, including IL-8, transforming growth factor (TGF)-β1, and neurotrophin-3, while others have found that the chemokine monocyte chemotactic protein-1 – also known as chemokine (C–C motif) ligand 2 – plays a role as a mediator for MSC migration to breast and colon cancers [53,54].

Because *in vitro* conditions often do not precisely mimic the *in vivo* tumor microenvironment, complex *in vivo* studies analyzing the effects of individual trophic factors have been undertaken by few investigators. In a study by Hata *et al*., U87 glioma cells were designed to secrete high or low amounts of PDGF-BB in an orthotopic mouse model of brain tumor [55]. Following intracarotid injection, human MSCs showed substantially enhanced homing into tumors expressing high levels of PDGF-BB compared with low-expressing tumors. Similarly, inhibiting the PDGF receptor on MSCs blocked the homing of MSCs to gliomas. These findings indicated that PDGF-BB plays a major role in MSC migration toward glioma in the *in vivo* setting. Similar *in vivo* experiments have implicated inflammation-derived factors, either directly or indirectly, in the recruitment and engraftment of MSCs [56]. A study by Shinojima *et al*. suggested that TGF-β1, which is released by many gliomas, and its

receptor TGF-βRII, which is present on MSCs, are involved in attracting MSCs to gliomas after the intravascular administration of MSCs [51]. Using a human glioma xenograft in an orthotopic mouse model, TGF-β was identified as a tumor-derived factor that attracts human MSCs through TGF-β type II and CD105 receptors on the cell surface. Using GICs isolated from patient tumors, this study demonstrated that intravascularly administered human MSCs are capable of homing to GICs, but only GICs that expressed a high level of TGF-β attracted human BM-MSCs [51].

Despite the many studies aimed at deciphering the homing mechanism of MSCs for gliomas and other tumors, much remains to be defined. It is unclear whether all of the known factors act independently or together, which factors initiate the homing of MSCs to gliomas, and which factors enhance the process. Moreover, after systemic administration, it is assumed that MSCs must interact with the endothelial cell "barrier" and then cross it. Factors mediating this interaction are largely unknown. MSCs, in particular, appear to home only to tumors and not to normal brain tissue, suggesting that the tumor's endothelial cells must harbor unique signals compared with normal brain endothelium.

## 60.7 Strategies to enhance mesenchymal stromal cell homing to gliomas

Understanding and teasing out the factors mediating MSC tropism toward gliomas provides immense opportunity to enhance this process and therefore to increase the delivery of therapeutic agents. Strategies aimed at increasing MSC tropism to gliomas generally involve either (1) driving the tumor to increase secretion of MSC-tropic factors, or (2) altering the MSCs to enhance their sensitivity to tumor-derived factors.

With regards to the first strategy, one way to increase MSC homing is to irradiate the tumor [57]. Radiation is thought to enhance and induce cell death and to release inflammatory molecules into the tumor microenvironment, which drive recruitment of MSCs that home to sites of tissue injury. Tumor irradiation was first shown to enhance the homing of MSCs to tumor tissue in a model of breast cancer [57]. The enhanced MSC tropism to the tumor was associated with the upregulation of local proinflammatory mediators including TGF-β1 and PDGF-BB [57]. Similar findings on the association between the inflammatory response and MSC tropism following radiation treatment were found in several experimental tumor models, including glioma and colon cancer [58,59]. Radiation of gliomas in one study resulted in both enhanced migration of MSCs to the tumor and upregulation of IL-8 and monocyte chemotactic protein-1 [58].

With regard to the second strategy involving altering the MSCs, several investigators have manipulated the surface proteins on MSCs to increase their responsiveness to tumor tropic factors. For example, Sato *et al.* demonstrated that mouse MSCs that are genetically engineered to overexpress the EGF receptor (EGFR) showed enhanced migratory responses toward syngeneic murine GL261 glioma or B16 melanoma models *in vivo* [60]. Moreover, intratumor injection of these tropism-enhanced MSCs engineered to secrete IFN-α resulted in significantly prolonged survival compared with nontropism-enhanced MSCs. A related strategy to increase MSC homing involves engineering MSCs to express an antibody toward a tumor-specific antigen on the tumor cell surface. Balyasnikova and coworkers engineered human MSCs to express an antibody to EGFR variant III (EGFRvIII), which enhanced human MSC homing toward glioma cells expressing EGFRvIII and this subsequently increased survival time in tumor-bearing mice [61,62].

An alternative strategy is to alter the MSC receptor profile by exposing MSCs to unique factors or conditions. For example, Shi *et al.* showed that treating MSCs with various cytokines upregulated chemokine and cytokine receptors on the MSC surface: pretreatment of MSCs with IL-6 or hepatocyte growth factor increased the expression of the chemokine receptor CXCR4, thereby augmenting MSC migration both *in vitro* and *in vivo* [63]. Similarly, placing MSCs in a hypoxic environment was also shown to promote MSC tropism to gliomas by promoting expression of the chemokine receptors CX3CR1 and CXCR4 on MSCs [64].

## 60.8 Types of therapeutic cargo

The unique tropism of MSCs for tumors makes them effective at transporting antitumor therapies. Although MSCs themselves could theoretically attack and inhibit tumors, the intrinsic antitumor properties of MSCs have only been shown in a few cancers [65–68]. Therefore, in MSC-based approaches, the antitumor cargo and its appropriateness are as critical to the efficacy of the approach as is the tumor-homing ability of the MSCs. Theoretically, the cargo must be able to kill or at least inhibit glioma cells. It must be able to be engineered into MSCs. It must not alter the tumor homing capacity of MSCs. It must not prematurely kill the MSC and, once within the tumor, the cargo must be released from the MSC. Based on these requirements, several types of antiglioma agents have been tested as cargoes and are broadly classified as secreted proteins, prodrug enzymes, oncolytic viruses, exosomes, and nanoparticles (Figure 60.4).

### 60.8.1 Secreted proteins

One broad class of antiglioma cargoes includes proteins secreted by MSCs. MSCs are typically engineered to produce these proteins by transducing the MSCs with a replication-incompetent viral vector carrying the gene encoding the protein or by transfection with a plasmid carrying the gene. The gene must then be transcribed and translated into the protein, and the protein must be secreted from the cell in a functional form in order affect the tumor. The MSC acts as a "mini-pump" of the secreted protein within the tumor. The longevity of most stromal cells in human tumors has not been well defined, but preclinical models suggest persistence of MSCs within a tumor for up to 3–4 weeks.

Proinflammatory cytokines are a group of secreted proteins that have been successfully delivered by MSCs in various glioma

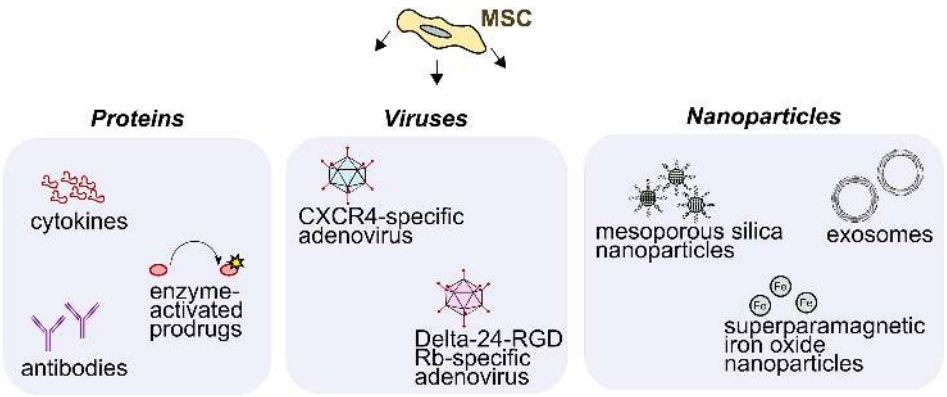



**Figure 60.4** MSCs can be engineered to release a variety of therapeutic cargo, including proteins, viruses, and nanoparticles.

model systems. Results, including *in vivo* outcomes, have illustrated the therapeutic potential of MSCs secreting recombinant modified IL-12M, which induces stronger type 1 T helper responses and antitumor immunity than wild-type IL-12. When injected directly into the tumor, mice receiving IL-12M-producing MSCs survived significantly longer than control mice. This result was explained by the induction of tumor-specific T-cell memory [69]. IFN-β is another cytokine known for its antiproliferative and proapoptotic effects in various cancers. Human MSCs engineered to express IFN-β were injected intravascularly into a human xenograft mouse model and resulted in decreased tumor growth and improved prognosis compared with control mice [7]. Furthermore, MSCs have been genetically modified to secrete other cytokines with substantial success: these include IL-2, IL-18, and IL-23 [17,70,71]. Cytokines have also demonstrated therapeutic efficacy when used in combination with other treatments, including short hairpin RNA, microRNA (miRNA), and the chemotherapeutic alkylating agent temozolomide [72–74].

Another secreted protein that has been engineered into MSCs is the proapoptotic protein tumor-necrosis-factor-related apoptosis-inducing ligand (TRAIL). TRAIL is a member of the tumor necrosis factor superfamily that induces apoptosis upon binding to its transmembrane receptors [75]. This particular receptor is absent in normal cells. Therefore, TRAIL is an attractive cancer therapeutic agent. Using mouse model systems of invasive human gliomas and utilizing real-time imaging with correlative neuropathology, Sasportas *et al.* demonstrated that MSCs engineered to express recombinant TRAIL or its secreted soluble variant (sTRAIL) had profound antitumor effects [76]. Additional studies demonstrated both therapeutic and tumor-specific effects after intracranial or intravenous injection of TRAIL- or sTRAIL-producing MSCs *in vivo* [76–78].

### 60.8.2 Prodrug enzymes

MSCs have also been engineered to release prodrug-activating enzymes that convert an inert prodrug into an active cytotoxic chemotherapeutic agent. The release of the active drug produces a bystander effect, killing the surrounding tumor cells. Because of their homing ability, MSCs deliver a local high-dose active chemotherapeutic agent to the tumor and a minimal dose, if any, to the peripheral organs, which avoids the systemic toxicity observed with many chemotherapeutic agents

The herpes simplex virus thymidine kinase (HSV-TK) enzyme/prodrug-activating gene therapy approach for treating malignant gliomas has been well characterized. HSV-TK is known as a suicide gene therapy because, on exposure to the prodrug ganciclovir, HSV-TK-transduced cells are killed and rapidly dividing bystander cells near them undergo apoptosis and are killed at the same time [79]. However, a phase III multicenter randomized clinical trial, which included glioblastoma patients, showed no significant difference in survival between the HSV-TK gene therapy group and controls [80]. The therapeutic failure was attributed to the low transduction efficiency of the drug to tumor cells and ineffective vector distribution within the tumors [80]. To rectify these problems, investigators loaded HSV-TK into MSCs as a way to more effectively deliver the drug to the tumor tissue. Murine studies using human glioma cell xenografts showed that HSV-TK-loaded MSCs enhanced prodrug activity through an HSV-TK-specific mechanism, leading to a better prognosis [24,81–83].

Several groups have developed MSCs engineered to express the rabbit carboxylesterase enzyme, which efficiently converts the prodrug CPT-11 (irinotecan) into its active anticancer form SN-38, a potent topoisomerase I inhibitor. Systemically administered (intracranially or intravenously) MSCs carrying rabbit carboxylesterase enzyme efficiently penetrated the brain and targeted both the primary glioma site and infiltrating glioma cells [84].

### 60.8.3 Replication-competent oncolytic viruses

Virotherapy involves genetically modifying viruses so that they will selectively infect and kill tumor cells. Although oncolytic virotherapy has delivered promising preclinical results, there are several obstacles to its successful clinical translation, including delivery and safety concerns. To address these problems several investigators used MSCs to deliver oncolytic viruses to the tumor by direct intratumor injection or by intravascular injection. Using the intravascular approach the MSCs are able to conceal the virus from the immune system as they home to the tumor. Once the MSCs infiltrate the tumor, the virus replicates within the MSCs, is released, and infects and destroys the surrounding tumor cells.

Sonabend *et al.* studied whether conditionally-replicating adenoviruses could be delivered by MSCs to gliomas [85]. Because the CXCR4 receptor is highly expressed on MSCs and glioma cells [85,86], they generated a virus with specificity for CXCR4. They found that this virus specifically infected and replicated within MSCs and glioma cells. This was the first report demonstrating that human MSCs could deliver oncolytic adenoviruses to malignant gliomas [87,88].

Delta-24-RGD is a novel oncolytic virus that is currently in clinical trials for patients with recurrent GBM. The Delta-24-RGD virus, also known as DNX-2401, exploits the fact that most glioma cells harbor deregulated retinoblastoma (Rb)-related proteins. Specifically, the Delta-24 virus contains a 24-base-pair deletion in the Early 1A adenoviral gene, the protein from which binds to the Rb protein. This virus can only replicate in cells lacking functioning Rb, and thus specifically in glioma cells [89]. Yong *et al.* loaded MSCs with Delta-24-RGD in an attempt to provide another viral-mediated method to target glioma cells. They showed that MSCs carrying Delta-24-RGD localized to human gliomas as effectively as uninfected MSCs did and that, once within the tumor, active virus was released from the MSCs, which then infected and replicated in the tumor cells. Analysis of tumor size showed that glioma growth was decreased and the tumor was even eradicated in nearly 40% of animals compared with controls [18]. Investigators in both conditionally-replicating adenoviruses and in Delta-24-RGD studies concluded that the intra-arterially delivered human MSC–oncolytic adenovirus selectively localized to human gliomas and delivered and released oncolytic adenovirus into the tumor, resulting in improved survival and tumor eradication *in vivo* [18,88].

### 60.8.4 Antibodies

Another approach to deliver antiglioma cargo by MSCs involves the use of antibodies. Using MSCs to deliver antitumor antibodies concentrates the distribution of the antibody to tumor tissue while sparing peripheral organs, therefore limiting cytotoxic off-target effects. EGFRvIII is a mutant form of EGFR that is found in nearly 50% of glioblastoma patients (reviewed by Heimberger *et al.* [90]). Several antibodies targeting EGFRs and EGFRvIIIs have been generated, and although *in vitro* results were encouraging, they exhibited poor results *in vivo* [91,92]. The dismal *in vivo* response was believed to result from low antibody penetration within the tumor tissue. To remedy this, Balyasnikova *et al.* sought to utilize MSCs as antibody delivery vehicles, which allowed for improved therapeutic penetrance. They demonstrated that co-injection of U87 human glioma cells and human MSCs transfected to express an antibody against the EGFRvIII resulted in reduced tumor vascularization and increased survival of glioma-bearing mice [61].

### 60.8.5 Nanoparticles

Nanoparticles can be either biological or synthetic materials that range from 1 to 100 nm in diameter. Historically, nanoparticles have had several limitations when administered systemically and have yielded conflicting therapeutic results. Systemically delivered nanoparticles are diluted by the presence of the BBB, so that their concentration within brain tumors is relatively low. To circumvent this problem investigators attempted to use cellular carriers, such as MSCs, as nanoparticle delivery vehicles to tumor tissue. For example, Roger *et al.* showed that polylactic acid or lipid nanoparticles could efficiently internalize into MSCs without affecting differentiation capabilities or cell viability [22]. *In vivo* studies showed that nanoparticle-loaded cells migrated toward human glioma xenografts [22]. Another study showed that super paramagnetic iron oxide nanoparticles could coat MSCs, which then successfully homed to brain tumors [93]. The ability of MSCs to home to gliomas while retaining mesoporous silica nanoparticles has also been evaluated. The loaded MSCs effectively homed to human orthotopic U87 tumor xenografts, whereas MSCs without nanoparticles did not home as well [94]. These findings demonstrated that MSC-secreting nanoparticles home to tumor tissue in the brain and therefore provide motivation for future applications of nanoparticle-mediated glioma therapeutics.

Exosomes are physiological nanoparticles (50–100 nm in diameter) that are secreted by cells and that have far-reaching biological effects (see Chapters 20 and 38). A host cell, such as an MSC, can release exosomes packaged with DNA, RNA, and protein into the extracellular space. They can then fuse with a target cell, releasing their contents into its cytoplasm. Exosomes thus behave as intracellular delivery systems. They can also be genetically engineered to express, and therefore transport, miRNA or small interfering RNA. MSC-derived exosomes have been used to treat human disease, including glioma. For example, direct intratumor injection of MSC-derived exosomes expressing miR-146 reduced glioma growth in a rat xenograft model [95]. Another group showed the therapeutic potential of delivering exosomes expressing miR-124 and miR-145 to human glioma stem cell xenograft tumors [96]. They confirmed that the synthetic miRNA was transferred by MSC exosomes to the target glioma cells. Furthermore, MSCs expressing high miR-124 levels were co-cultured with glioma U87 cell lines and were subsequently found to decrease U87 cell migration. Finally, they showed that MSCs could deliver miR-124 to U87 human glioma xenografts *in vivo*, by measuring the down regulation of miR-124 target genes in glioma tissues [96]. However, whether delivery of these miRNAs promoted survival or induced tumor regression was not reported, suggesting the need for more studies to validate the therapeutic efficacy of this approach.

## 60.9 Delivery routes of mesenchymal stromal cells in clinical applications

The route of administration represents a critical variable for the therapeutic delivery of MSCs. As implied earlier, several strategies for MSC transplantation in GBM have been proposed and tested. Local intratumor injection of MSCs directly into the tumor was the original strategy proposed. This approach maximally exploits

the capacity of MSCs to migrate throughout a tumor with the goal of reaching infiltrating tumor cells. The advantage of the approach is that all the cells are deposited directly into the tumor. On the other hand, this method is highly invasive as it requires passing a needle into the brain and tumor via a burr hole or twist drill, which carries the potential risk of brain injury and hemorrhage. The approach also requires that MSCs are able to travel long distances from the injection site, which may be difficult in large, highly infiltrative tumors. Multiple injections can overcome this shortcoming, but each injection carries a risk of hemorrhage.

Another difficulty with intratumoral injection is that the method can be cumbersome after tumor resection. In this situation the MSCs often flow out of the injection site along a path of least resistance into the resection cavity, from which they are then washed away. To overcome this problem, and to increase the survival of transplanted MSCs, Shah *et al.* reported the development of a hydrogel in which to encapsulate the MSCs prior to implantation. This method was found to significantly improve the therapeutic efficacy of transplanted MSCs in various glioma models (reviewed in [97]).

Another attractive method of administration is systemic delivery into the bloodstream. Systemic delivery has the advantage that it is a minimally invasive approach, and therefore multiple injections over time are possible. Moreover, in theory the MSCs should be able to access all areas of the tumor via the vessel portals, potentially producing a more widespread initial deposition of MSCs compared with intratumor injection of the cells. Although there are studies promoting the ability of MSCs to localize to brain tumors after intravenous injection, most investigators report that intravenously infused cells are typically held up in the pulmonary capillary bed and only a reduced number reach their target site. Consequently, preclinical studies and proposed clinical applications have relied on intra-arterial injection because it eliminates lung entrapment. With modern endovascular techniques, including super selective catheterization of intracranial vessels, intra-arterial delivery is a viable option in clinical practice and has been applied in regenerative medicine approaches [98].

MSCs have also been delivered to the brain intranasally. This particular technique was first applied to the treatment of neurodegenerative disorders [99] and was then assessed in glioma [100]. Specifically, investigators showed that MSCs delivered intranasally were able to infiltrate the brain through the nasal cavity and migrate toward glioma xenografts [100]. Furthermore, Balyasnikova *et al.* showed that intranasal delivery of MSCs was a therapeutically relevant way to deliver antiglioma agents, as MSCs carrying sTRAIL prolonged the survival of glioma-bearing mice after intranasal delivery [101].

## 60.10 Mesenchymal stem cells in the biology of gliomas

The capacity of exogenous MSCs to home to tumors led to the concept that endogenous MSCs may also be attracted into tumors and may, therefore, contribute to the tumor microenvironment. This concept gained further traction when it was found that, in addition to their residence in the bone marrow, MSCs also reside within most organs of the body as a type of pericyte in the endothelial wall. Therefore, at least theoretically, MSCs can be attracted into tumors not only from distant bone marrow sources but also from local organ-resident pools.

## 60.11 Controversy over tumor-associated mesenchymal stromal cells in solid tumors and gliomas

Investigators have begun to explore the contribution of MSCs to the tumor microenvironment [30,102–105]. Along with endothelial cells, immune cells, and fibroblasts, increasing evidence suggests that MSCs also contribute to the stroma of many solid tumors as so-called tumor-associated MSCs (TA-MSCs), due to their natural tropism for tumors [36,37]. Although some studies suggest that MSCs inhibit tumor growth [28], most studies demonstrate that MSCs enhance tumor growth and invasion, facilitate resistance to therapy, and contribute to the establishment of distant metastases in multiple cancer types [106–111]. In a seminal paper, MSCs were shown to modulate the invasive and metastatic nature of breast cancer tumors through the induction of chemokine (C–C motif) ligand 5 secretion and the upregulation of chemokine receptor 5 in cancer cells [112]. In ovarian cancer, TA-MSCs have been shown to facilitate proliferation and metastatic spread and have also been shown to serve a protective role against hyperthermic intraperitoneal chemotherapy mediated by the secretion of specific proteins, IL-6, IL-8, and C–X–C motif chemokine ligand 12 [113,114]. TA-MSCs were also found to significantly have an impact on the proliferation, migration, and invasion of pancreatic carcinoma and to decrease the efficacy of chemoradiation therapy [115]. In colorectal carcinoma, TA-MSCs were determined to play an oxidative role in the tumor microenvironment by recycling by-products of anaerobic metabolism in order to sustain hypoxic cancer cells [116]. Lastly, prostate cancer cells were shown to increase their growth and undergo histologic changes in response to TA-MSC challenges [117].

In brain tumors, however, the understanding of the interaction between the tumor and its microenvironment is still incomplete. The microenvironment of GBMs has traditionally been thought to consist of reactive astrocytes, vascular cells, microglia, immune cells, and neural progenitor cells [118]. However, several studies have suggested that MSC-like cells may also contribute to the stroma of human gliomas [119,120]. Indeed, MSCs have been shown to reside in normal brain around blood vessels [121–123], and therefore it is likely that MSCs are recruited into developing brain tumors.

Consistent with these concepts, our group has recently isolated cells that have a phenotype similar to BM-MSCs from glioblastoma surgical specimens. We have called these cells glioma-associated MSCs (GA-MSCs). Like BM-MSCs, and as defined by

the International Society of Cellular Therapy, the isolated GA-MSCs (1) grow with a spindle-shaped morphology on plastic, (2) express the characteristic MSC surface markers CD105, CD73, and CD90 and do not express markers of the hematopoietic and immune systems including CD45, CD34, and T cell and B cell markers [124], and (3) differentiate into osteocytes, adipocytes, and chondrocytes when exposed to the appropriate induction medium *in vitro* [125]. Immunohistochemical analyses of GBMs suggest that these GA-MSCs occur both around vessels in the tumor and within the tumor mass *per se*.

Equally importantly, we have found that these human GA-MSCs are not bystanders within the tumor, but like TA-MSCs in other tumors, contribute to the biological behavior of GICs, which maintain and drive glioma growth. Specifically, using *in vitro* co-culture methods we have shown that GA-MSCs increase the proliferation and clonogenicity of GICs. Using *in vivo* assays we have also shown that GA-MSCs increased the tumor-forming capacity of GICs and enhanced the aggressive behavior of GICs, as demonstrated by shortened animal survival. As in other solid tumors, the effects of human GA-MSCs on GICs are due at least in part to a direct paracrine influence of GA-MSCs on the tumor cells. Specifically, we found that that the increased proliferation effect was mediated at least in part by the GA-MSCs' secretion of IL-6, which drives proliferation and stemness of GICs by activating the signal transducer and activator of transcription 3.

## 60.12 A model of mesenchymal stromal cells in glioma biology

Taken together, these results suggest that human GA-MSCs may be a heretofore unrecognized component of the complex microenvironment of human gliomas (Figure 60.5).

It is proposed that GICs secrete factors that attract MSCs to gliomas. These MSCs may come from the bone marrow or from local perivascular reservoirs in the brain. Once within the tumor, the GICs may commandeer the MSCs, driving them to become GA-MSCs, which then engage in crosstalk with the GICs. This results in the secretion of tumor-promoting factors by the GA-MSCs that help drive tumor growth and proliferation. Complete analysis of this hypothesis remains an active area of research. Nevertheless, these data suggest that interfering with the attraction of MSCs to gliomas or disrupting the crosstalk between GICs and GA-MSCs may thwart the growth of gliomas and may represent a new approach to targeting glioma growth.

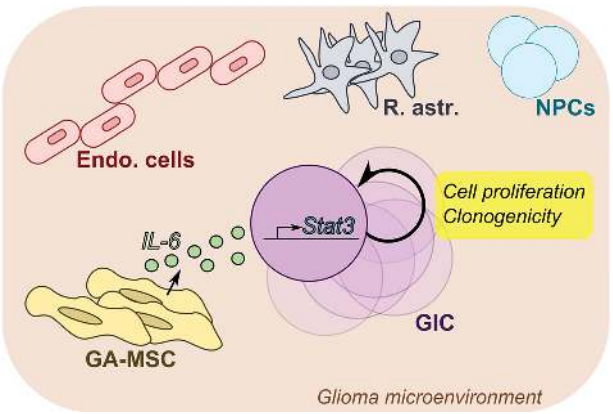



**Figure 60.5** GA-MSCs influence the glioma microenvironment. GA-MSCs release IL-6, which activates signal transducer and activator of transcription 3 (Stat3) signaling in GICs. Endo: endothelial; NPCs: neural progenitor cells; R. astr: reactive astrocytes. (See color plate section.)

## 60.13 Prospects for clinical use of bone-marrow-derived mesenchymal stromal cells in glioma therapy

MSCs are currently being investigated in several clinical trials for a large number of diseases, including musculoskeletal disorders, neurodegenerative disease, and spinal cord injuries. In fact, according to ClinicalTrials.gov, there were 491 trials listed when the database was searched with the term "mesenchymal stem cell" and 96 when the term "mesenchymal stromal cell" was used as of April 2015. However, there were only two clinical trials using MSCs as vehicles to deliver anticancer agents to tumors, and neither of these were in brain tumors. In one clinical trial, MSCs were being used to deliver an oncolytic adenovirus to recurrent ovarian cancer tumors refractory to standard treatments (NCT02068794). In another clinical trial, MSCs were being used to deliver IL-12 in order to activate natural killer and cytotoxic T lymphocytes in patients with head and neck tumors (NCT02079324).

Despite the large literature on MSCs in cancer therapy, it is disappointing that there are so few clinical trials at this time. Potential reasons for trepidation in the field include apprehension that cultured MSCs may themselves be tumorigenic. This concern has stemmed from rare reports suggesting the instability of MSCs *in vitro* [126–128]. Clearly, an important factor for using MSCs as therapeutic delivery vehicles is their ability to be expanded *in vitro* without undergoing genetic transformation. Unfortunately, several reports suggest that murine MSCs may display suboptimal stability *in vitro* [126,127], and have even exhibited the capability of forming sarcomas in animal models *in vivo* [129]. However, in contrast to murine MSCs, the majority of studies on human MSCs have confirmed their stability [130]. Nevertheless, these studies emphasize the importance of stringent monitoring of the stability of MSCs in culture, including karyotyping before their use in human studies. In general, if MSCs are used clinically only at early passages (less than four or five passages) the risk of tumorigenicity appears to be low.

Another cause for concern is the potential of MSCs to increase tumor growth and aggressiveness. As discussed earlier, the role of MSCs in tumor progression has been documented in several tumor types, including gliomas [112,131,132]. MSCs appear to drive tumor growth in large part by secreting an array of

tumor-promoting factors. Therefore, it is critical that any therapeutic approach involves engineering MSCs so that they are driven to secrete high levels of antiglioma factors, thereby subverting their normal secretory repertoire, which may drive GIC stemness and proliferation. Thus, delivery of "empty" MSCs must be scrutinized. It is imperative that MSCs are robustly engineered so that the majority of delivered MSCs actually contain the antiglioma agent. Another option involves loading MSCs with cargoes containing agents that destroy the MSCs once within the tumor. For this approach, delivering oncolytic viruses is particularly attractive because the release of virus from the MSC results in lysis of the MSC. Of course, a safety valve could be built into MSCs by engineering a self-destruct system, such as HSV-TK. This would allow the MSCs to secrete an antiglioma agent of choice until the cells are exposed to the antiviral agent ganciclovir, which kills MSCs expressing the transduced viral thymidine kinase but does not kill cells that contain the gene for human thymidine kinase. As in all fields, it is imperative for translational researchers to develop and further advance clinical approaches that are grounded in strong preclinical science and that are both efficacious and safe.

## References


1 Stupp R, Mason WP, van den Bent MJ, *et al*. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 2005;**352**(10):987–996.

2 Friedenstein AJ, Chailakhyan RK, Latsinik NV, *et al*. Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues. Cloning *in vitro* and retransplantation *in vivo*. *Transplantation* 1974;**17**(4):331–340.

3 Zuk PA, Zhu M, Ashjian P, *et al*. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**(12):4279–4295.

4 Lee OK, Kuo TK, Chen WM, *et al*. Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* 2004;**103**(5):1669–1675.

5 Crisan M, Chen C-W, Corselli M, *et al*. Perivascular multipotent progenitor cells in human organs. *Ann N Y Acad Sci* 2009;**1176**: 118–123.

6 Dominici M, Le Blanc K, Mueller I, *et al*. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.

7 Nakamizo A, Marini F, Amano T, *et al*. Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;**65**(8):3307–3318.

8 Tabatabai G, Bahr O, Mohle R, *et al*. Lessons from the bone marrow: how malignant glioma cells attract adult haematopoietic progenitor cells. *Brain* 2005;**128**(Pt 9):2200–2211.

9 Ferrari N, Glod J, Lee J, *et al*. Bone marrow-derived, endothelial progenitor-like cells as angiogenesis-selective gene-targeting vectors. *Gene Ther* 2003;**10**(8):647–656.

10 Aboody KS, Brown A, Rainov NG, *et al*. Neural stem cells display extensive tropism for pathology in adult brain: evidence from intracranial gliomas. *Proc Natl Acad Sci U S A* 2000;**97**(23): 12846–12851.

11 Levison SW, Goldman JE. Multipotential and lineage restricted precursors coexist in the mammalian perinatal subventricular zone. *J Neurosci Res* 1997;**48**(2):83–94.

12 Cai J, Wu Y, Mirua T, *et al*. Properties of a fetal multipotent neural stem cell (NEP cell). *Dev Biol* 2002;**251**(2):221–240.

13 Eriksson PS, Perfilieva E, Bjork-Eriksson T, *et al*. Neurogenesis in the adult human hippocampus. *Nat Med* 1998;**4**(11):1313–1317.

14 Johansson CB, Momma S, Clarke DL, *et al*. Identification of a neural stem cell in the adult mammalian central nervous system. *Cell* 1999;**96**(1):25–34.

15 Hatten ME. Central nervous system neuronal migration. *Annu Rev Neurosci* 1999;**22**:511–539.

16 Ehtesham M, Kabos P, Kabosova A, *et al*. The use of interleukin 12-secreting neural stem cells for the treatment of intracranial glioma. *Cancer Res* 2002;**62**(20):5657–5663.

17 Nakamura K, Ito Y, Kawano Y, *et al*. Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004;**11**(14):1155–1164.

18 Yong RL, Shinojima N, Fueyo J, *et al*. Human bone marrow-derived mesenchymal stem cells for intravascular delivery of oncolytic adenovirus Delta24-RGD to human gliomas. *Cancer Res*. 2009;**69**(23):8932–8940.

19 Uzzaman M, Keller G, Germano IM. *In vivo* gene delivery by embryonic-stem-cell-derived astrocytes for malignant gliomas. *Neuro Oncol* 2009;**11**(2):102–108.

20 Aboody KS, Najbauer J, Metz MZ, *et al*. Neural stem cell-mediated enzyme/prodrug therapy for glioma: preclinical studies. *Sci Transl Med* 2013;**5**(184): 184ra59.

21 Van Eekelen M, Sasportas LS, Kasmieh R, *et al*. Human stem cells expressing novel TSP-1 variant have anti-angiogenic effect on brain tumors. *Oncogene* 2010;**29**(22):3185–3195.

22 Roger M, Clavreul A, Venier-Julienne MC, *et al*. Mesenchymal stem cells as cellular vehicles for delivery of nanoparticles to brain tumors. *Biomaterials* 2010;**31**(32):8393–8401.

23 Lee EX, Lam DH, Wu C, *et al*. Glioma gene therapy using induced pluripotent stem cell derived neural stem cells. *Mol Pharm* 2011;**8** (5):1515–1524.

24 Ryu CH, Park KY, Kim SM, *et al*. Valproic acid enhances anti-tumor effect of mesenchymal stem cell mediated HSV-TK gene therapy in intracranial glioma. *Biochem Biophys Res Commun* 2012;**421**(3):585–590.

25 Gage FH. Mammalian neural stem cells. *Science* 2000;**287** (5457):1433–1438.

26 Houghton J, Stoicov C, Nomura S, *et al*. Gastric cancer originating from bone marrow-derived cells. *Science* 2004;**306**(5701): 1568–1571.

27 Caplan AI. Why are MSCs therapeutic? *New data: new insight. J Pathol* 2009;**217**(2):318–324.

28 Djouad F, Plence P, Bony C, *et al*. Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. *Blood* 2003;**102**(10):3837–3844.

29 Beckermann BM, Kallifatidis G, Groth A, *et al*. VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. *Br J Cancer* 2008;**99**(4):622–631.

30 Coffelt SB, Marini FC, Watson K, *et al*. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through

recruitment of multipotent mesenchymal stromal cells. *Proc Natl Acad Sci U S A* 2009;**106**(10):3806–3811.
31 Grinnemo KH, Mansson A, Dellgren G, *et al.* Xenoreactivity and engraftment of human mesenchymal stem cells transplanted into infarcted rat myocardium. *J Thorac Cardiovasc Surg* 2004;**127**(5): 1293–1300.
32 Dezawa M, Ishikawa H, Itokazu Y, *et al.* Bone marrow stromal cells generate muscle cells and repair muscle degeneration. *Science* 2005;**309**(5732):314–317.
33 Chen J, Li Y, Wang L, *et al.* Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci* 2001;**189**(1–2):49–57.
34 Li Y, Chen J, Wang L, *et al.* Intracerebral transplantation of bone marrow stromal cells in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease. *Neurosci Lett* 2001;**316**(2):67–70.
35 Studeny M, Marini FC, Champlin RE, *et al.* Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. *Cancer Res* 2002;**62**(13):3603–3608.
36 Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;**315**(26):1650–1659.
37 Duan X, Guan H, Cao Y, Kleinerman ES. Murine bone marrow-derived mesenchymal stem cells as vehicles for interleukin-12 gene delivery into Ewing sarcoma tumors. *Cancer* 2009;**115**(1):13–22.
38 Xiang J, Tang J, Song C, *et al.* Mesenchymal stem cells as a gene therapy carrier for treatment of fibrosarcoma. *Cytotherapy* 2009;**11** (5):516–526.
39 Kanehira M, Xin H, Hoshino K, *et al.* Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. *Cancer Gene Ther* 2007;**14**(11):894–903.
40 Hung SC, Deng WP, Yang WK, *et al.* Mesenchymal stem cell targeting of microscopic tumors and tumor stroma development monitored by noninvasive *in vivo* positron emission tomography imaging. *Clin Cancer Res* 2005;**11**(21):7749–7756.
41 Komarova S, Kawakami Y, Stoff-Khalili MA, *et al.* Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* 2006;**5**(3):755–766.
42 Kallifatidis G, Beckermann BM, Groth A, *et al.* Improved lentiviral transduction of human mesenchymal stem cells for therapeutic intervention in pancreatic cancer. *Cancer Gene Ther* 2008;**15** (4):231–240.
43 Gao P, Ding Q, Wu Z, *et al.* Therapeutic potential of human mesenchymal stem cells producing IL-12 in a mouse xenograft model of renal cell carcinoma. *Cancer Lett* 2010;**290**(2):157–166.
44 Hall B, Dembinski J, Sasser AK, *et al.* Mesenchymal stem cells in cancer: tumor-associated fibroblasts and cell-based delivery vehicles. *Int J Hematol* 2007;**86**(1):8–16.
45 Doucette T, Rao G, Yang Y, *et al.* Mesenchymal stem cells display tumor-specific tropism in an RCAS/Ntv-a glioma model. *Neoplasia* 2011;**13**(8):716–725.
46 Fisher GH, Orsulic S, Holland E, *et al.* Development of a flexible and specific gene delivery system for production of murine tumor models. *Oncogene* 1999;**18**(38):5253–5260.
47 Holland EC, Hively WP, DePinho RA, Varmus HE. A constitutively active epidermal growth factor receptor cooperates with disruption of $G_1$ cell-cycle arrest pathways to induce glioma-like lesions in mice. *Genes Dev* 1998;**12**(23):3675–3685.
48 Dunlap SM, Celestino J, Wang H, *et al.* Insulin-like growth factor binding protein 2 promotes glioma development and progression. *Proc Natl Acad Sci U S A* 2007;**104**(28):11736–11741.
49 Singh SK, Clarke ID, Terasaki M, *et al.* Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003;**63**(18):5821–5828.
50 Galli R, Binda E, Orfanelli U, *et al.* Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004;**64**(19):7011–7021.
51 Shinojima N, Hossain A, Takezaki T, *et al.* TGF-β Mediates homing of bone marrow-derived human mesenchymal stem cells to glioma stem cells. *Cancer Res* 2013;**73**(7):2333–2344.
52 Birnbaum T, Roider J, Schankin CJ, *et al.* Malignant gliomas actively recruit bone marrow stromal cells by secreting angiogenic cytokines. *J Neurooncol* 2007;**83**(3):241–247.
53 Menon LG, Picinich S, Koneru R, *et al.* Differential gene expression associated with migration of mesenchymal stem cells to conditioned medium from tumor cells or bone marrow cells. *Stem Cells* 2007;**25**(2):520–528.
54 Dwyer RM, Potter-Beirne SM, Harrington KA, *et al.* Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clin Cancer Res* 2007;**13** (17):5020–5027.
55 Hata N, Shinojima N, Gumin J, *et al.* Platelet-derived growth factor BB mediates the tropism of human mesenchymal stem cells for malignant gliomas. *Neurosurgery* 2010;**66**(1):144–156, discussion 156–157.
56 Spaeth E, Klopp A, Dembinski J, *et al.* Inflammation and tumor microenvironments: defining the migratory itinerary of mesenchymal stem cells. *Gene Ther* 2008;**15**(10):730–738.
57 Klopp AH, Spaeth EL, Dembinski JL, *et al.* Tumor irradiation increases the recruitment of circulating mesenchymal stem cells into the tumor microenvironment. *Cancer Res* 2007;**67**(24):11687–11695.
58 Kim SM, Oh JH, Park SA, *et al.* Irradiation enhances the tumor tropism and therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand-secreting human umbilical cord blood-derived mesenchymal stem cells in glioma therapy. *Stem Cells* 2010;**28**(12):2217–2228.
59 Zielske SP, Livant DL, Lawrence TS. Radiation increases invasion of gene-modified mesenchymal stem cells into tumors. *Int J Radiat Oncol Biol Phys* 2009;**75**(3):843–853.
60 Sato H, Kuwashima N, Sakaida T, *et al.* Epidermal growth factor receptor-transfected bone marrow stromal cells exhibit enhanced migratory response and therapeutic potential against murine brain tumors. *Cancer Gene Ther* 2005;**12**(9):757–768.
61 Balyasnikova IV, Ferguson SD, Sengupta S, *et al.* Mesenchymal stem cells modified with a single-chain antibody against EGFRvIII successfully inhibit the growth of human xenograft malignant glioma. *PLoS ONE* 2010;**5**(3):e9750.
62 Balyasnikova IV, Franco-Gou R, Mathis JM, Lesniak MS. Genetic modification of mesenchymal stem cells to express a single-chain antibody against EGFRvIII on the cell surface. *J Tissue Eng Regen Med* 2010;**4**(4):247–258.
63 Shi M, Li J, Liao L, *et al.* Regulation of CXCR4 expression in human mesenchymal stem cells by cytokine treatment: role in homing efficiency in NOD/SCID mice. *Haematologica* 2007;**92** (7):897–904.
64 Hung SC, Pochampally RR, Hsu SC, *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression

of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* 2007;**2**(5):e416.

65 Glass R, Synowitz M, Kronenberg G, *et al*. Glioblastoma-induced attraction of endogenous neural precursor cells is associated with improved survival. *J Neurosci* 2005;**25**(10):2637–2646.

66 Cousin B, Ravet E, Poglio S, *et al*. Adult stromal cells derived from human adipose tissue provoke pancreatic cancer cell death both *in vitro* and *in vivo*. *PLoS ONE* 2009;**4**(7):e6278.

67 Secchiero P, Zorzet S, Tripodo C, *et al*. Human bone marrow mesenchymal stem cells display anti-cancer activity in SCID mice bearing disseminated non-Hodgkin's lymphoma xenografts. *PLoS ONE* 2010;**5**(6):e11140.

68 Khakoo AY, Pati S, Anderson SA, *et al*. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *J Exp Med* 2006;**203**(5):1235–1247.

69 Ryu CH, Park SH, Park SA, *et al*. Gene therapy of intracranial glioma using interleukin 12-secreting human umbilical cord blood-derived mesenchymal stem cells. *Hum Gene Ther* 2011;**22**(6): 733–743.

70 Yuan X, Hu J, Belladonna ML, *et al*. Interleukin-23-expressing bone marrow-derived neural stem-like cells exhibit antitumor activity against intracranial glioma. *Cancer Res* 2006;**66**(5):2630–2638.

71 Xu G, Jiang XD, Xu Y, *et al*. Adenoviral-mediated interleukin-18 expression in mesenchymal stem cells effectively suppresses the growth of glioma in rats. *Cell Biol Int* 2009;**33**(4):466–474.

72 Kock N, Kasmieh R, Weissleder R, Shah K. Tumor therapy mediated by lentiviral expression of shBcl-2 and S-TRAIL. *Neoplasia* 2007;**9**(5):435–442.

73 Corsten MF, Miranda R, Kasmieh R, *et al*. MicroRNA-21 knockdown disrupts glioma growth *in vivo* and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas. *Cancer Res* 2007;**67**(19):8994–9000.

74 Hingtgen S, Ren X, Terwilliger E, *et al*. Targeting multiple pathways in gliomas with stem cell and viral delivered S-TRAIL and temozolomide. *Mol Cancer Ther* 2008;**7**(11):3575–3585.

75 Schneider P, Olson D, Tardivel A, *et al*. Identification of a new murine tumor necrosis factor receptor locus that contains two novel murine receptors for tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). *J Biol Chem* 2003;**278**(7):5444–5454.

76 Sasportas LS, Kasmieh R, Wakimoto H, *et al*. Assessment of therapeutic efficacy and fate of engineered human mesenchymal stem cells for cancer therapy. *Proc Natl Acad Sci U S A* 2009;**106** (12):4822–4827.

77 Kim SK, Kim SU, Park IH, *et al*. Human neural stem cells target experimental intracranial medulloblastoma and deliver a therapeutic gene leading to tumor regression. *Clin Cancer Res* 2006;**12** (18):5550–5556.

78 Menon LG, Kelly K, Yang HW, *et al*. Human bone marrow-derived mesenchymal stromal cells expressing S-TRAIL as a cellular delivery vehicle for human glioma therapy. *Stem Cells* 2009;**27** (9):2320–2330.

79 Van Dillen IJ, Mulder NH, Vaalburg W, *et al*. Influence of the bystander effect on HSV-tk/GCV gene therapy. *A review. Curr Gene Ther* 2002;**2**(3):307–322.

80 Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000;**11** (17):2389–2401.

81 Miletic H, Fischer Y, Litwak S, *et al*. Bystander killing of malignant glioma by bone marrow-derived tumor-infiltrating progenitor cells expressing a suicide gene. *Mol Ther* 2007;**15**(7):1373–1381.

82 Uchibori R, Okada T, Ito T, *et al*. Retroviral vector-producing mesenchymal stem cells for targeted suicide cancer gene therapy. *J Gene Med* 2009;**11**(5):373–381.

83 Matuskova M, Hlubinova K, Pastorakova A, *et al*. HSV-tk expressing mesenchymal stem cells exert bystander effect on human glioblastoma cells. *Cancer Lett* 2010;**290**(1):58–67.

84 Choi SA, Lee JY, Wang KC, *et al*. Human adipose tissue-derived mesenchymal stem cells: characteristics and therapeutic potential as cellular vehicles for prodrug gene therapy against brainstem gliomas. *Eur J Cancer* 2012;**48**(1):129–137.

85 Lopez P, Machado M, Zamprogno H, *et al*. Phenotypic characterization of canine bone marrow stromal cells. *J Stem Cells Regen Med* 2007;**2**(1):178–179.

86 Son BR, Marquez-Curtis LA, Kucia M, *et al*. Migration of bone marrow and cord blood mesenchymal stem cells *in vitro* is regulated by stromal-derived factor-1–CXCR4 and hepatocyte growth factor–c-met axes and involves matrix metalloproteinases. *Stem Cells* 2006;**24**(5):1254–1264.

87 Sonabend AM, Ulasov IV, Han Y, *et al*. Biodistribution of an oncolytic adenovirus after intracranial injection in permissive animals: a comparative study of Syrian hamsters and cotton rats. *Cancer Gene Ther* 2009;**16**(4):362–372.

88 Sonabend AM, Ulasov IV, Tyler MA, *et al*. Mesenchymal stem cells effectively deliver an oncolytic adenovirus to intracranial glioma. *Stem Cells* 2008;**26**(3):831–841.

89 Fueyo J, Gomez-Manzano C, Alemany R, *et al*. A mutant oncolytic adenovirus targeting the Rb pathway produces anti-glioma effect *in vivo*. *Oncogene* 2000;**19**(1):2–12.

90 Heimberger AB, Suki D, Yang D, *et al*. The natural history of EGFR and EGFRvIII in glioblastoma patients. *J Transl Med* 2005;**3**:38.

91 Patel D, Lahiji A, Patel S, *et al*. Monoclonal antibody cetuximab binds to and down-regulates constitutively activated epidermal growth factor receptor vIII on the cell surface. *Anticancer Res* 2007;**27**(5A):3355–3366.

92 Neyns B, Sadones J, Joosens E, *et al*. Stratified phase II trial of cetuximab in patients with recurrent high-grade glioma. *Ann Oncol* 2009;**20**(9):1596–1603.

93 Wu X, Hu J, Zhou L, *et al*. *In vivo* tracking of superparamagnetic iron oxide nanoparticle-labeled mesenchymal stem cell tropism to malignant gliomas using magnetic resonance imaging. *Laboratory investigation. J Neurosurg* 2008;**108**(2):320–329.

94 Huang X, Zhang F, Wang H, *et al*. Mesenchymal stem cell-based cell engineering with multifunctional mesoporous silica nanoparticles for tumor delivery. *Biomaterials* 2013;**34**(7):1772–1780.

95 Katakowski M, Buller B, Zheng X, *et al*. Exosomes from marrow stromal cells expressing miR-146b inhibit glioma growth. *Cancer Lett* 2013;**335**(1):201–204.

96 Lee HK, Finniss S, Cazacu S, *et al*. Mesenchymal stem cells deliver synthetic microRNA mimics to glioma cells and glioma stem cells and inhibit their cell migration and self-renewal. *Oncotarget* 2013;**4** (2):346–361.

97 Shah K. Encapsulated stem cells for cancer therapy. *Biomatter* 2013;**3**(1):e24278. doi: 10. 4161/biom.24278.

98 Lee PH, Lee JE, Kim HS, *et al.* A randomized trial of mesenchymal stem cells in multiple system atrophy. *Ann Neurol* 2012;**72**(1): 32–40.

99 Danielyan L, Schafer R, von Ameln-Mayerhofer A, *et al.* Therapeutic efficacy of intranasally delivered mesenchymal stem cells in a rat model of Parkinson disease. *Rejuvenation Res* 2011;**14**(1):3–16.

100 Reitz M, Demestre M, Sedlacik J, *et al.* Intranasal delivery of neural stem/progenitor cells: a noninvasive passage to target intracerebral glioma. *Stem Cells Transl Med* 2012;**1**(12):866–873.

101 Balyasnikova IV, Prasol MS, Ferguson SD, *et al.* Intranasal delivery of mesenchymal stem cells significantly extends survival of irradiated mice with experimental brain tumors. *Mol Ther* 2014;**22**(1): 140–148.

102 Liu S, Ginestier C, Ou SJ, *et al.* Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* 2011;**71**(2):614–624.

103 McLean K, Gong Y, Choi Y, *et al.* Human ovarian carcinoma-associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. *J Clin Invest* 2011;**121** (8):3206–3219.

104 Yan XL, Fu CJ, Chen L, *et al.* Mesenchymal stem cells from primary breast cancer tissue promote cancer proliferation and enhance mammosphere formation partially via EGF/EGFR/Akt pathway. *Breast Cancer Res Treat* 2011;**132**(1):153–164.

105 Luo J, Ok Lee S, Liang L, *et al.* Infiltrating bone marrow mesenchymal stem cells increase prostate cancer stem cell population and metastatic ability via secreting cytokines to suppress androgen receptor signaling. *Oncogene* 2014;**33**(21):2768–2678.

106 Zhu W, Xu W, Jiang R, *et al.* Mesenchymal stem cells derived from bone marrow favor tumor cell growth *in vivo*. *Exp Mol Pathol* 2006;**80**(3):267–274.

107 Muehlberg FL, Song YH, Krohn A, *et al.* Tissue-resident stem cells promote breast cancer growth and metastasis. *Carcinogenesis* 2009;**30**(4):589–597.

108 Kucerova L, Matuskova M, Hlubinova K, *et al.* Tumor cell behaviour modulation by mesenchymal stromal cells. *Mol Cancer* 2010;**9**:129.

109 Shinagawa K, Kitadai Y, Tanaka M, *et al.* Mesenchymal stem cells enhance growth and metastasis of colon cancer. *Int J Cancer* 2010;**127**(10):2323–2333.

110 Suzuki K, Sun R, Origuchi M, *et al.* Mesenchymal stromal cells promote tumor growth through the enhancement of neovascularization. *Mol Med* 2011;**17**(7–8):579–587.

111 Goldstein RH, Reagan MR, Anderson K, *et al.* Human bone marrow-derived MSCs can home to orthotopic breast cancer tumors and promote bone metastasis. *Cancer Res* 2010;**70** (24):10044–10050.

112 Karnoub AE, Dash AB, Vo AP, *et al.* Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449** (7162):557–563.

113 Lis R, Touboul C, Mirshahi P, *et al.* Tumor associated mesenchymal stem cells protects ovarian cancer cells from hyperthermia through CXCL12. *Int J Cancer* 2011;**128**(3):715–725.

114 Spaeth EL, Dembinski JL, Sasser AK, *et al.* Mesenchymal stem cell transition to tumor-associated fibroblasts contributes to fibrovascular network expansion and tumor progression. *PLoS ONE* 2009;**4**(4):e4992.

115 Hwang RF, Moore T, Arumugam T, *et al.* Cancer-associated stromal fibroblasts promote pancreatic tumor progression. *Cancer Res* 2008;**68**(3):918–926.

116 Koukourakis MI, Giatromanolaki A, Harris AL, Sivridis E. Comparison of metabolic pathways between cancer cells and stromal cells in colorectal carcinomas: a metabolic survival role for tumor-associated stroma. *Cancer Res* 2006;**66**(2):632–637.

117 Olumi AF, Grossfeld GD, Hayward SW, *et al.* Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 1999;**59**(19):5002–5011.

118 Charles NA, Holland EC, Gilbertson R, *et al.* The brain tumor microenvironment. *Glia* 2012;**60**(3):502–514.

119 Tso CL, Shintaku P, Chen J, *et al.* Primary glioblastomas express mesenchymal stem-like properties. *Mol Cancer Res* 2006;**4**(9): 607–619.

120 Kim SM, Kang SG, Park NR, *et al.* Presence of glioma stroma mesenchymal stem cells in a murine orthotopic glioma model. *Childs Nerv Syst* 2011;**27**(6):911–922.

121 Kang SG, Shinojima N, Hossain A, *et al.* Isolation and perivascular localization of mesenchymal stem cells from mouse brain. *Neurosurgery* 2010;**67**(3):711–720.

122 Crisan M, Yap S, Casteilla L, *et al.* A perivascular origin for mesenchymal stem cells in multiple human organs. *Cell Stem Cell* 2008;**3**(3):301–313.

123 Da Silva Meirelles L, Chagastelles PC, Nardi NB. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *J Cell Sci* 2006;**119**(Pt 11):2204–2213.

124 Bianco P, Robey PG, Simmons PJ. Mesenchymal stem cells: revisiting history, concepts, and assays. *Cell Stem Cell* 2008;**2** (4):313–319.

125 Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411): 143–147.

126 Miura M, Miura Y, Padilla-Nash HM, *et al.* Accumulated chromosomal instability in murine bone marrow mesenchymal stem cells leads to malignant transformation. *Stem Cells* 2006;**24**(4): 1095–1103.

127 Zhou YF, Bosch-Marce M, Okuyama H, *et al.* Spontaneous transformation of cultured mouse bone marrow-derived stromal cells. *Cancer Res* 2006;**66**(22):10849–10854.

128 Wang Y, Huso DL, Harrington J, *et al.* Outgrowth of a transformed cell population derived from normal human BM mesenchymal stem cell culture. *Cytotherapy* 2005;**7**(6):509–519.

129 Tolar J, Nauta AJ, Osborn MJ, *et al.* Sarcoma derived from cultured mesenchymal stem cells. *Stem Cells* 2007;**25**(2):371–379.

130 Bernardo ME, Zaffaroni N, Novara F, *et al.* Human bone marrow derived mesenchymal stem cells do not undergo transformation after long-term *in vitro* culture and do not exhibit telomere maintenance mechanisms. *Cancer Res* 2007;**67**(19):9142–9149.

131 Zhu W, Huang L, Li Y, *et al.* Mesenchymal stem cell-secreted soluble signaling molecules potentiate tumor growth. *Cell Cycle* 2011;**10**(18):3198–3207.

132 Zhu W, Huang L, Li Y, *et al.* Exosomes derived from human bone marrow mesenchymal stem cells promote tumor growth *in vivo*. *Cancer Lett* 2012;**315**(1):28–37.

CHAPTER 61

# Mesenchymal stromal cells as gene delivery vehicles to treat nonmalignant diseases

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## 61.1 Introduction

Mesenchymal stromal cells (MSCs) are excellent candidates for cellular therapies and are often investigated for regenerative medicine applications. The multipotent status of these cells makes them prime candidates for tissue regeneration, whereby they can directly differentiate and replace damaged tissues. The ability of MSCs to differentiate into cells of mesodermal lineage, including osteoblasts, adipocytes, and chondrocytes, is well documented [1]. Furthermore, MSCs release a variety of cytokines, chemokines and other soluble factors in response to injury [2] (reviewed by Phinney and Prockop [3]). This paradigm, where MSCs may be utilized to promote tissue healing through long-term release of soluble factors rather than by replacing cells in damaged tissues, demonstrates the importance of developing gene-modified MSCs so that we may take advantage of, and even accentuate, the cells' endogenous responses. A phase-contrast image of MSCs in culture is shown in Figure 61.1.

The ease of isolation and expansion, interaction with the immune system, and migratory capabilities of MSCs also make them an ideal cell source for genetic modification to combine the best of both cell and gene therapy for a multitude of diverse diseases. Efficacy has been demonstrated in preclinical and clinical studies using MSCs for cardiovascular, musculoskeletal, neurodegenerative, and autoimmune diseases and in acute graft-versus-host disease (GVHD) [4–6]. These and other extensive studies have thoroughly demonstrated the safety of MSCs for clinical use [7].

## 61.2 What are mesenchymal stromal cells?

MSCs were first isolated from bone marrow by Freidenstein *et al.* in 1970 based on their ability to adhere to plastic [8]. Since then MSCs have been found in many other tissues, including adipose tissue, brain, liver, lung, kidney, and dental pulp [8,9]. They can also be found in umbilical cord blood and amniotic fluid [10,11]. Though MSCs from each of these tissues appear phenotypically similar, cells from each tissue have subtle molecular differences indicative of their tissue of origin [12–14]. Freidenstein *et al.* originally characterized these cells as multipotent for stromal precursors and clonogenic in culture [8]. Differentiation of MSCs into other cell types, such as cardiomyocytes [15], neurons [16], and hepatocytes [17], have also been described in the literature.

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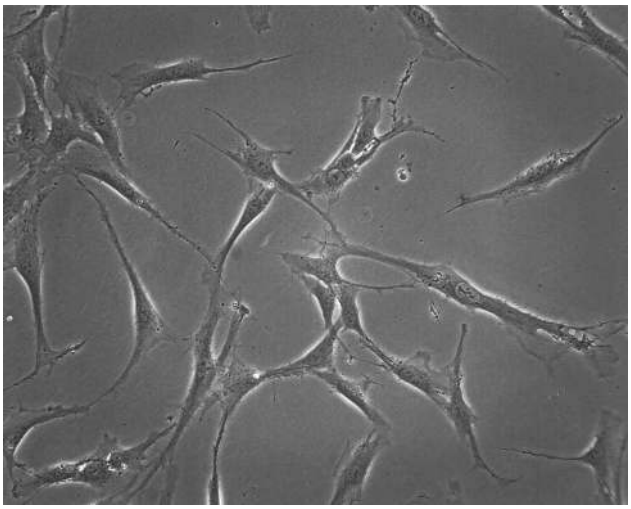


**Figure 61.1** A phase-contrast image of human bone marrow-derived mesenchymal stem/stromal cells in culture.

However, the ability of MSCs to differentiate into nonmesodermal lineage cell types is hotly debated: cells may be forced to differentiate into a specific lineage and express appropriate surface markers in culture, but do not necessarily display characteristic functions of that cell type.

The precise definition of MSCs continues to be disputed. The International Society for Cell Therapy currently defines human multipotent MSCs as possessing the following criteria: (1) MSCs are adherent to plastic when cultured in standard conditions; (2) MSCs express CD105, CD73, and CD90 and lack expression of CD45, CD34, CD14 or CD11b, CD79-α, CD19, and HLA-DR surface molecules; and (3) MSCs must differentiate into osteoblasts, adipocytes, and chondroblasts *in vitro* [18]. It is important to note that these requirements are specifically for human MSCs and do not wholly translate to other animal sources of MSCs.

## 61.3 Genetic modification of mesenchymal stromal cells

### 61.3.1 Safety concerns

There have been numerous studies demonstrating the clinical safety of MSCs [7]. Since the first clinical trial utilizing MSCs in 1995 testing the feasibility of extracting, expanding, and administering MSCs in humans [19], both allogeneic and autologous MSCs have been given to thousands of patients to treat various diseases, including bone and cartilage defects, cardiovascular diseases, autoimmune diseases, neurological diseases, and refractory wounds [20–22].

The clinical use of gene therapy in humans, however, still faces many challenges. Of the 440 clinical trials utilizing MSCs currently listed on clinicaltrials.gov, very few are utilizing gene-modified cells. Considering the drawbacks and limited success seen in early gene therapy clinical trials, it is not surprising that progress has been slow in this field, despite its vast potential. A patient enrolled in a trial aimed at evaluating the safety of gene therapy for ornithine transcarboxylase deficiency died because of a severe immune response to the gene-delivering viral vector [23]. In clinical studies to deliver interleukin (IL)-2 to X-linked severe combined immunodeficiency patients, initial promising results were overshadowed by patients who subsequently developed leukemia-like disorders and died due to insertional mutagenesis caused by the incorporation of the IL-2 gene retroviral vector near an oncogene of the delivered cells [23]. In 2003 the use of retroviral vectors in blood stem cells was temporarily suspended by the US Food and Drug Administration, with ongoing trials placed on clinical hold. Years later, when the factors contributing to insertional mutagenesis from retroviral vectors were better understood and the risk versus benefit ratio was better defined in each trial, some were allowed to continue.

Owing to the significant safety concerns of genetic modification, other methods of delivering growth factors, enzymes, and other therapeutically relevant signals have been explored. Direct growth factor supplementation therapy has been explored in the clinic with an inadequate result due to several limitations: because of the rate at which growth factors are broken down or excreted, they must be administered frequently and in high doses to initiate tissue healing [24]. The costly and transitory nature of this approach is far from ideal. Direct administration of gene-expressing plasmids has also been investigated. For example, Ye *et al.* administered systemically naked plasmid containing a deficient clotting factor, factor IX (FIX), in a murine model of hemophilia B. They observed therapeutic levels of human FIX for a year after plasmid administration, but treated animals also exhibited acute hepatic damage due to the high concentration of viral plasmid [25]. MSCs modified to express FIX in a controlled manner may be a safer approach to address the factor deficiency of this factor.

Significant advances have been made to understand the tumor induction capacity of the genetically modified cells used in previous trials [26,27]. Based on the results of Koegel *et al.*, self-inactivating lentiviral vectors have been developed, effectively eliminating the major tumorigenic component caused by using retroviral plasmids for future gene-modified stem cell trials [27,28]. Furthermore, MSCs have not been reported to cause *in vivo* tumor formation. We and others have demonstrated biosafety of retro- and lentivirally transduced MSCs with extensive decade-long studies using sensitive xenograft assays [29].

### 61.3.2 Choice of vector system

MSCs can be genetically engineered by permanent integration of the gene of choice, or through episomal expression of the gene by viral vector transduction or transient expression of nonvirally delivered genes [30]. (An episome is a genetic particle within cells that can exist either autonomously in the cytoplasm or as part of a chromosome). Viral delivery of specific genes to MSCs is the

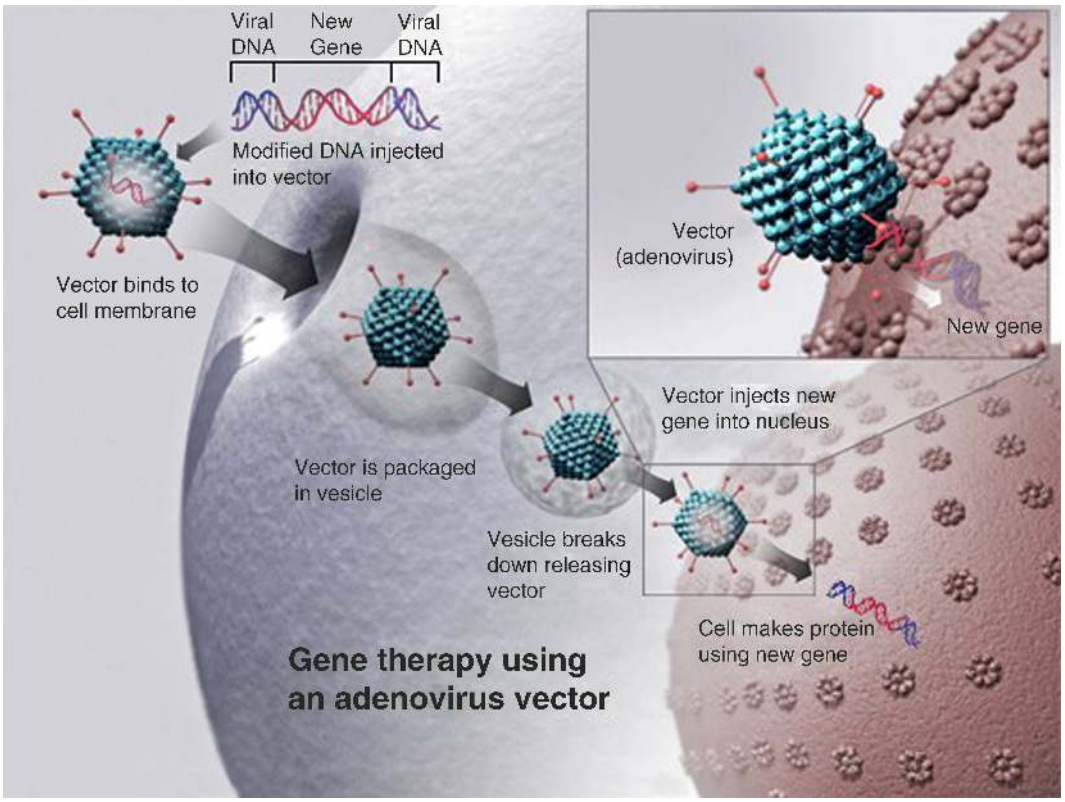



**Figure 61.2** A new gene is inserted into a cell using an adenovirus. (See color plate section.) *Source:* http://commons.wikimedia.org/wiki/File:Gene_therapy.jpg#/media/File:Gene_therapy.jpg

most common method in preclinical research, but the transduction efficiency depends on the target cells [31]. Transduced cells can contain thousands of copies of the transgene RNA per cell and maintain expression for over 6 months [4,32].

Retroviral, lentiviral, adenoviral, and adeno-associated virus vectors have been used in gene-modified MSC studies [30]. Episomal expression of transgenes can be achieved through the use of adenoviral and adeno-associated virus vectors and can sustain long-term transgene expression in non-dividing cells. However, as cells proliferate, transgene expression from episomal vectors becomes diluted and diminishes. In addition, capsids of adenoviral vectors can prompt an immune response, leading to adverse events [33]. Therefore, this approach may be best suited for applications in which short-term transgene expression is desired, such as for increasing homing and cell survival of transplanted cells. Figure 61.2 depicts the use of an adenoviral vector for gene transfer into a cell.

For sustained transgene expression, retroviral and lentiviral vectors are ideal. Lentiviral vectors are able to transduce nondividing cells, while retroviral vectors cannot. Previous work has indicated that MSCs can be stably transduced with both retroviral and lentiviral vectors and maintain differentiation potential and *in vivo* engraftment with no adverse events, even after multiple passages [29,32]. Figure 61.3 depicts the packaging and transduction of a gene using a lentivirus vector.

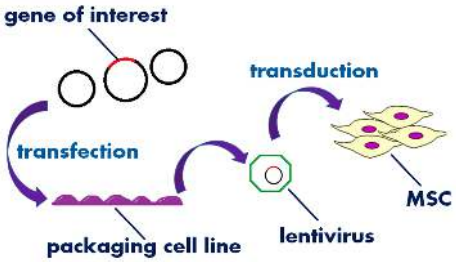



**Figure 61.3** Schematic overview of lentivirus generation and genetic modification of MSCs. To modify MSCs to express a gene of interest, a packaging cell line is first transfected with DNA plasmids containing the gene of interest and other elements for lentivirus production. Within 3 days the transfected packaging cells produce lentivirus containing the gene of interest, which is then used to transduce MSCs, which will then express the gene of interest. This image was generated by F. Fierro.

## 61.4 Preclinical models of gene-modified mesenchymal stromal cells: mesenchymal stromal cell migration and survival

MSCs are known to migrate to areas of tissue damage (see Chapters 22 and 23). The number of MSCs increases during total body hypoxia or irradiation, implying that they are part of

an innate healing response [34,35] (see Chapter 16). Additionally, MSCs have similar morphology and express similar markers to pericytes, which are cells that surround blood vessels throughout the body [11]. It has been hypothesized that MSCs are pericytes, and are therefore naturally located for easy access to circulating cytokines and chemokines, and with a means to migrate in response to trafficking signals. These endogenous trafficking signals can be utilized to promote migration of systemically administered cells to the location of tissue injury. MSCs express a variety of chemokine receptors including CCR1, CCR7, CCR9, CXCR3, CXCR4, CXCR5, and CXCR1 [36] (and see Chapter 23). Specific chemokine–receptor pairs shown to mediate MSC migration include hepatocyte growth factor (HGF) and its receptor c-MET, vascular endothelial growth factor (VEGF)/VEGF receptor, monocyte chemoattractant protein 1 (also known as CCL2)/CCR2, and others (reviewed by Karp and Leng Teo [37]).

Unfortunately, despite the preferential homing ability of MSCs to sites of inflammation, poor engraftment of the cells after administration has been unexpected and has given rise to the paracrine hypothesis of how MSCs may mediate their actions (see Chapters 21 and 37). One reason for this limited channeling of cells to target tissues is the large proportion of intravenously administered MSCs that become lodged in filtering organs, particularly the lungs, but also the liver, kidney, and spleen [37,38]. Other delivery methods, such as intra-arterial, intraperitoneal, or intracardiac injection, may lead to higher rates of engraftment but are more invasive [39]. Sasaki *et al.* were able to overcome low migration of MSCs in a mouse model of dermal wound healing by intradermally injecting the CCR7 ligand CCL21 (also known as the secondary lymphoid-tissue chemokine (SLC)) [40]. They found that MSC-treated animals exhibited rapid wound repair compared with control animals, but wound repair in SLC/CCL21-treated animals was even further accelerated, reportedly due to increased MSC migration to the wound site.

Another potential explanation for poor migration and engraftment of MSCs after administration may be that, while homing-specific chemokine receptors are upregulated in areas of tissue damage [41,42], *in vitro* expanded MSCs express low levels of some key chemokine receptors [43,44]. MSCs exposed to hypoxia *in vitro*, however, can induce expression of the most critical chemokine receptor CXCR4 [45] (and see Chapter 16). Systemically administered MSCs modified to overexpress CXCR4 showed increased migration to the infarct region in a rat model of acute myocardial infarction (AMI), resulting in a decrease of abnormal ventricular remodeling [46]. Furthermore, location of engrafted CXCR4-overexpressing MSCs in the infarcted heart was associated with increased angiogenesis and myogenesis [47]. Administration of these modified MSCs also demonstrated positive results in murine models of acute liver failure and traumatic brain injury [48,49]. MSCs overexpressing stromal-derived factor-1 (also known as CXCL12 and the ligand of CXCR4) increased bone formation in a rat model of fracture repair [50]. Endogenous CD44 on MSCs was engineered to function as the ligand for E-selectin, a cell adhesion molecule that is constitutively expressed only on endothelial cells of specialized marrow vessels and which is essential for cell trafficking within the bone marrow. Sackstein *et al.* demonstrated that this modification was sufficient to induce homing of MSCs to bone marrow [51].

Intramyocardially injected MSCs modified to overexpress CCR1 were shown to accumulate in infarcted myocardium after coronary artery ligation in mice and to contribute to restored cardiac function 4 weeks after AMI, possibly due to the higher expression of the CCR1 ligand, CCL7, in infarcted heart muscle [52]. A schema for the mechanism of an acute myocardial infarct is shown in Figure 61.4.

Adipose-tissue-derived MSCs modified to overexpress granulocyte chemotactic protein (GCP)-2 (also known as CXCL6) showed higher engraftment after intramyocardial injection, improved cardiac function, and reduced infarct size in a murine model of AMI. The investigators found that these cells also expressed significantly higher levels of VEGF, HGF, IL-8, insulin-like growth factor 1 (IGF-1), and AKT, suggesting that adipose-tissue-derived MSCs genetically altered to overexpress GCP-2 had enhanced angiogenic and survival properties [53].

In ischemic diseases, hypoxia is a critical factor that challenges the survival of cells after transplantation. Some strategies that have been employed to adapt the cells to this harsh environment include genetic modification for overexpression of endogenous hypoxia-regulated and prosurvival signals. Heme oxygenase-1 (HO-1), a stress-inducible enzyme, has been reported to have a cytoprotective role when administered alone [54]. MSCs modified to over-express HO-1 (HO-1-MSCs) have demonstrated protection against apoptosis in several animal models of cardiac ischemia [55–58]. Tang *et al.* demonstrated that HO-1-MSCs exhibited increased cell survival, enhanced functional recovery, and decreased pathological left ventricular remodeling in a murine model of AMI [59]. Furthermore, HO-1-MSCs showed enhanced expression of VEGF and fibroblast growth factor (FGF)-2, indicating that HO-1 overexpression can promote cell survival and therapeutic angiogenesis of MSCs in an ischemic setting [58].

The inflammatory nature of damaged tissues is also a challenge to the survival of MSCs after administration. MSCs have been modified to overexpress antiapoptotic molecules such as B cell lymphoma-2, erythropoietin (EPO), survivin, HSP-20 (heat shock protein with a molecular weight of 20 kDa), and connexin 43 for use in animal models of AMI [60–64]. Animals treated with MSCs modified to overexpress these prosurvival genes did indeed exhibit increased survival, as well as increased capillary density in infarcted regions. (Survivin is a member of the inhibitor of apoptosis family. The survivin protein functions to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. HSPs are a family of proteins that are produced by cells in response to exposure to stressful conditions. They were first described in relation to heat shock, but are now

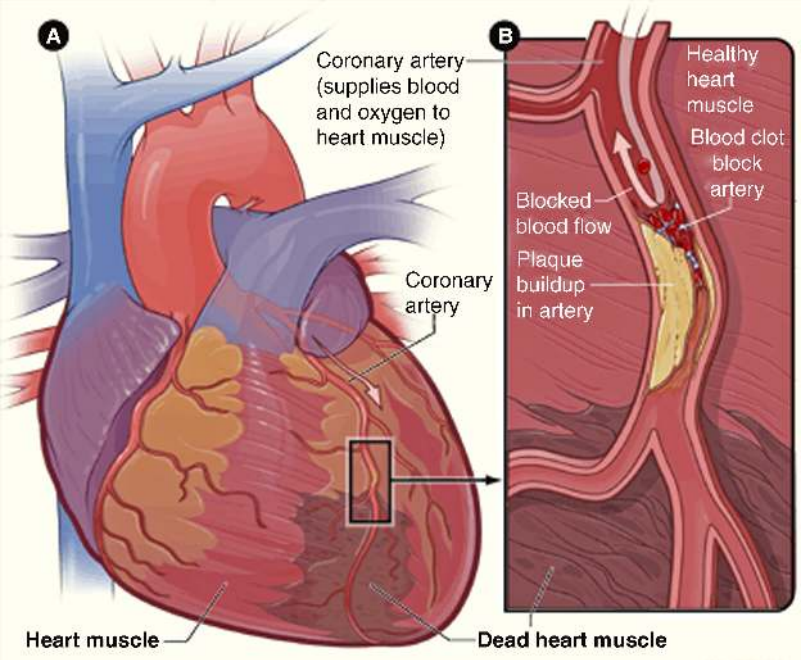



**Figure 61.4** Diagram showing an AMI (heart attack). *Source:* nih.gov.

known to also be expressed during other stresses, including exposure to cold, UV light, and during wound healing or tissue remodeling. Connexins, or gap junction proteins, are a family of structurally related transmembrane proteins that assemble to form vertebrate gap junctions. Each gap junction is composed of two hemichannels, or connexons, which are themselves each constructed out of six connexin molecules. Gap junctions are essential for many physiological processes, such as the coordinated depolarization of cardiac muscle, proper embryonic development, and the conducted response in microvasculature).

Positive results associated with these gene-modified MSCs were linked to enhanced *AKT* activation and secretion of growth factors [64].

Because of its role in multiple cellular processes, including cell survival and proliferation, *AKT1* (the gene that encodes the AKT protein) is a popular target for increasing MSC survival [65]. Mangi *et al.* found a significant increase in retained AKT-MSCs (MSCs modified to express AKT) after myocardial injection into ischemic rat hearts compared with unmodified MSCs. Animals treated with AKT-MSCs also exhibited reduced intramyocardial inflammation and collagen deposition in a dose-dependent manner and regenerated 80–90% of lost myocardial volume [66]. Similar results were reported by a different group utilizing AKT-MSCs in a swine myocardial infarction model [67]. We have demonstrated that exposing MSCs to 3% oxygen for 24 h also upregulates AKT activity and enhances their homing, survival, and revascularization potential [45]. Conditioned medium from MSCs exposed to hypoxia has shown protective effects on survival of adult rat ventricular cardiomyocytes subjected to hypoxia *in vitro*, compared with conditioned medium from MSCs without hypoxic exposure. However, the greatest MSC-mediated cytoprotective effects were seen when rat ventricular cardiomyocytes were exposed to conditioned medium from hypoxic MSCs that were also genetically modified to overexpress AKT [68].

Other growth factors have also been utilized to promote the survival of transplanted MSCs. Among the most studied is VEGF. MSCs transduced with the VEGF gene have been studied in multiple animal models of myocardial infarction [69–72] and critical limb ischemia with good outcomes [32]. Hepatocyte growth factor (HGF) has also proved promising as a candidate for genetic modification of MSCs in animal models of cardiovascular disease [72–75]. Fully elucidating the mechanisms responsible for MSC survival and migration/homing will be critical in future therapeutic applications of these cells.

## 61.5 Gene-modified mesenchymal stromal cells as immune modulators

MSCs are thought to play a critical role in hematopoietic homeostasis in the bone marrow (reviewed by Pontikoglou *et al.* [76] – and see Chapter 32). MSCs are able to avoid detection and resulting clearance by the recipient's immune system due, at least in part, to their lack of expression of major histocompatibility complex (MHC) class II antigens and the costimulatory molecules CD80 and CD86 [77]. This makes MSCs attractive candidates for allogeneic cell therapy. MHC-unmatched MSCs can be administered without eliciting a host immune response [78] (but see Chapter 29). Furthermore, MSCs have an ability to exert immunomodulatory and anti-inflammatory effects [79] (and see Chapters 33 and 34). Through a

combination of cell-to-cell contact and secretion of soluble factors, MSCs can induce division arrest in T cells, B cells, natural killer cells, and dendritic cells. They can also inhibit the cytotoxicity of T cells and natural killer cells as well as affecting B cell and dendritic cell function, effectively crippling the adaptive immune system (reviewed by Vetyska and Krusina [80] – and Chapter 34).

The precise mechanisms for many of the immunomodulatory interactions of MSCs are subject to debate, but evidence suggests many are largely dependent on paracrine signaling. MSCs are known to express a multitude of immune modulating factors, such as prostaglandin-$E_2$ [81], nitric oxide [82], HGF [83], transforming growth factor-β1 [84], and indoleamine 2,3-dioxygenase [85]. The potential beneficial effects of the immune modulating properties of MSCs can be exploited in many disease models to reduce local inflammation and recruitment of immune cells to the area of tissue injury. For example, Granero-Moltó *et al.* reported a decrease in local and circulating levels of the proinflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, and IL-6 after MSC transplantation in a murine tibial fracture model [86]. Multiple studies have utilized MSCs as an adjunct to allogeneic hematopoietic stem cell transplantation in order to prevent graft rejection or to treat GVHD. The latter is a common complication of allogeneic hematopoietic stem cell transplantation in which donor T cells mount an immune response to the recipient's tissues that bear minor histocompatibility antigens not shared by the donor, in spite of matching between donor and recipient for the antigens of the MHC and in spite of the routine prophylactic use of medications such as cyclosporine, methotrexate, tacrolimus, or rapamycin [87–90]. Additional immune suppressive agents, such as corticosteroids, can be used to treat GVHD, but they render the patient even more vulnerable to infection [91]. In 2004, Le Blanc *et al.* successfully treated a patient with severe acute GVHD refractory to conventional immune suppressive medications using *ex vivo* expanded but not gene-modified maternal (haploidentical) MSCs [92]. Other clinical trials have demonstrated that co-transplantation of MSCs with allogeneic HSC transplants significantly reduced acute GVHD [92–94].

The anti-inflammatory effects of MSCs can be amplified by genetically modifying specific immunomodulatory signals that are specific to certain pathologies. For example, MSCs modified with angiotensin-converting enzyme 2 (ACE2) – an enzyme that degrades angiotensin II, which is involved in endothelial dysfunction during acute lung injury – have demonstrated beneficial anti-inflammatory effects *in vitro*. He *et al.* found that MSCs modified to express ACE2 decreased production of inflammatory mediators and enhanced survival of endothelial cells after lipopolysaccharide-induced cell injury in tissue culture [95]. MSCs modified to express angiopoietin-1 have also been utilized in a mouse model of acute lung injury to decrease damage and scarring by inhibition of recruitment of inflammatory cells [96]. Inhibition of TNF-α, an inflammatory mediator involved in ischemia–reperfusion injury, may produce beneficial effects in ischemic disease [97]. Bao *et al.* [98] utilized TNF receptor (TNFR)-modified MSCs (TNFR-MSCs) in a rat model of myocardial infarction with the hypothesis that TNFR-MSCs would bind and remove TNF-α from the microenvironment. They found that TNFR-MSC-treated animals had improved left ventricular function and increased MSC engraftment in the infarcted myocardium compared with control animals [98].

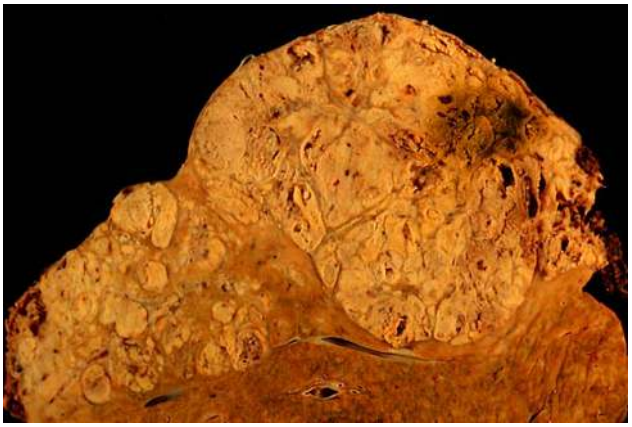


**Figure 61.5** Hepatic cirrhosis and hepatocellular carcinoma. Histopathologic image of an autopsy specimen from a person with cirrhosis of the liver in which a hepatocellular carcinoma had developed. There is a rim of fibrotic cirrhotic tissue at the bottom of the image, while the greater part of the image shows the hepatocellular carcinoma. *Source:* http://commons.wikimedia.org/wiki/File:Hepatocellular_carcinoma_1.jpg#/media/File:Hepatocellular_carcinoma_1.jpg

Fiore *et al.* exposed primary cultured hepatocytes to IGF-1-modified MSC supernatant, which induced increased expression of IGF-1 and HGF in the cultured cells. They then systemically administered IGF-1-MSCs in a murine model of cirrhosis and found decreased liver fibrosis and an increased expression of proliferating cell nuclear antigen in hepatocytes of IGF-1-MSC-treated animals [99]. Cirrhosis can cause death either by liver failure or by predisposing to hepatocellular carcinoma (Figure 61.5).

MSCs modified to express the gene for HGF in a rat model of allograft liver transplantation significantly increased survival time compared with animals treated with unmodified MSCs. This improvement in survival correlated with decreased IL-2 and interferon-α, increased IL-4 and IL-10, as well as reduced apoptosis and increased liver regeneration [100].

## 61.6 Gene-modified mesenchymal stromal cells in skeletal disorders

Chronic musculoskeletal pain has been reported as the most common medical complaint in the USA. Timely healing of damaged bone and cartilage by innate mechanisms is hampered by lack of local progenitor cells and adequate signaling. MSCs are promising candidates for musculoskeletal regeneration primarily owing to their ability to differentiate into osteoblasts and

chondrocytes. Indeed, one of the first clinical trials utilizing MSCs was in the treatment of osteogenesis imperfecta (OI), a genetic disease in which osteocytes produce defective type I collagen, causing bones to be extremely fragile [5]. MSCs can also be forced into tenocyte differentiation *in vitro* for transplant therapy and have been used in equine models of tendon repair [101]. Beyond their capacity to differentiate and replace damaged tissue, the ability of MSCs to secrete bioactive factors that alter the local environment and influence resident progenitor cells has also proven advantageous for musculoskeletal therapies. The combination of cell and gene therapy is ideal for bone and cartilage healing applications, whereby cell differentiation and signaling can be directed at the site of tissue injury.

The bone morphogenetic protein (BMP) family of growth factors has been widely utilized for the genetic modification of MSCs for musculoskeletal disorders. BMPs regulate a diverse set of biological processes, including bone and cartilage formation [102]. Multiple BMP family members, including BMB2, -4, and -7, have been examined for bone regenerating therapies in several animal models [103]. For example, Tsuji *et al.* found that BMP2 signaling is necessary for initiation of the fracture-healing process in mice [104]. Clinical trials utilizing BMP2 alone for tibial and spinal nonunion fractures have been conducted with promising results [105]. However, one group found that high doses of BMPs are required for efficacy, and may still not be effective, as is the case with many growth factor supplementation strategies [24]. It has been robustly demonstrated that MSCs modified to overexpress BMP2 (BMP2-MSCs) can form ectopic bone in mice [106–108], and that MSCs do not need to differentiate into osteoblasts to express BMPs [109]. As discussed earlier, MSCs are able to home and engraft to fracture sites [86] to exert their reparative effects at the site of tissue damage. In a rat articular fracture model, BMP2-MSCs induced bone and cartilage healing after direct cell injection [110].

BMP2-MSCs seem an ideal candidate for fracture therapy. BMP4- and BMP7-MSCs have also shown bone-inducing potential in animal models of osteoporosis and cranial bone defects [111–113]. BMP7 has been implicated as a candidate for the genetic modification of MSCs to increase chondrogenesis in an *in vitro* model of chondrocyte transplantation [114]. Modification of MSCs with BMP12 can induce *in vitro* tenocyte differentiation [115]. However, the tissue source of MSCs may also be an important factor for BMP-MSC-based therapies. Park *et al.* examined adipose-tissue-, bone-marrow-, and periosteal-derived MSCs after stimulation with BMP2 in a rat model of articular cartilage repair. They found that bone marrow and periosteal MSCs demonstrated increased cartilage repair when compared with adipose-derived MSCs, but BMP2 was required for initiation of chondrogenesis with all cell types [116].

Basic FGF (bFGF), sonic hedgehog, WNT4, LIM mineralization protein, and IGF-1 have all been implicated as candidates for modification of MSCs, each independently demonstrating increased efficacy in skeletal disease models over unmodified MSCs [117–125]. However, bFGF-MSCs and unmodified MSCs were tested in a rat model of Achilles tendon defect with negligible effects in either group [126].

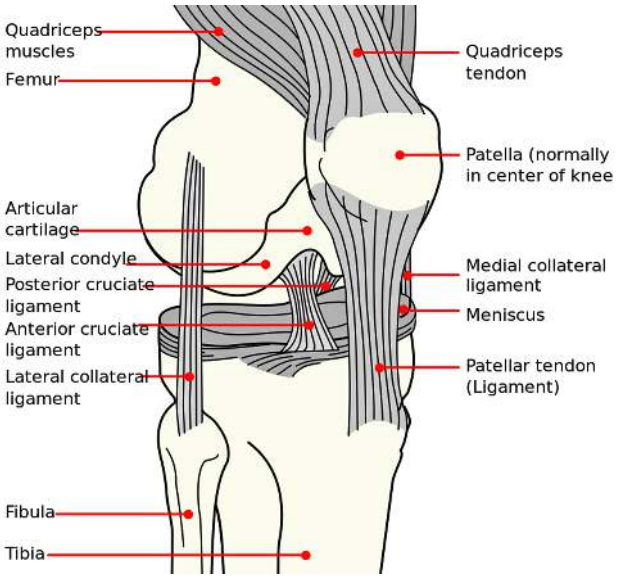



**Figure 61.6** The anatomical structure of the right human knee showing the muscles and ligaments associated with it, including the ACL. *Source:* http://commons.wikimedia.org/wiki/File:Knee_diagram.svg#/media/File:Knee_diagram.svg

Membrane type 1 matrix-metalloproteinase-modified MSCs demonstrated reduced scar formation and improved biomechanical strength in a rat model of rotator cuff healing [127]. Ectopic cartilage formation was achieved in pigs, rabbits, and rats utilizing transforming growth factor (TGF)-β1-MSCs [128–131]. Li *et al.* utilized unmodified MSCs and MSCs modified to overexpress platelet-derived growth factor (PDGF)-B for anterior cruciate ligament (ACL) reconstruction in a rabbit and found that in both cell-treated groups cellular infiltration was increased in the ACL. However, PDGF-B-MSCs induced initial angiogenesis that was not seen in the MSC group [132]. Figure 61.6 shows a diagram of the human knee, giving the anatomical position of the ACL.

RUNX2, a transcription factor that is important in osteogenesis and chondrogenesis, has also been utilized for gene therapy applications in musculoskeletal diseases. RUNX2-MSCs transplanted in a murine calvaria (skull bone) defect model showed increased bone mineral density and bone volume at the site of the defect compared with unmodified MSCs [133]. Other groups have modified MSCs to overexpress human telomerase reverse transcriptase to determine whether replicative senescence can be prevented to increase osteogenic potential of the cells [134,135].

As with bone repair, transcription factors can also regulate chondrogenic pathways: SOX9, Brachyury (which regulates FGFR3 and BMP2), and Sprouty1 have been investigated as potential targets for modification of MSCs in cartilage repair models [136–138]. MSCs modified to overexpress scleraxis, a transcription factor that regulates tendon development during




**Figure 61.7** A photograph of a child with the rickets-like changes that occur in hypophosphatasia. The image shows the typical widening of the wrists due to cupping and fraying of the metaphyseal region where there is an excess of nonmineralized osteoid tissue resulting in growth plate widening. *Source:* http://commons.wikimedia.org/wiki/File:Rickets_wrist.jpg#/media/File:Rickets_wrist.jpg.

embryogenesis, induced rotator cuff healing in rats at an earlier time point than unmodified MSCs did [139].

Monogenic bone diseases, such as hypophosphatasia and OI are ideal models for MSC-mediated gene therapy. As the name implies, hypophosphatasia is a condition in which tissue nonspecific alkaline phosphatase (ALP) is genetically inactivated in osteoblasts and chondrocytes [140]. As a result, bones exhibit reduced mineralization, eventually leading to rachitic changes (Figure 61.7) and osteomalacia.

Infants suffering with hypophosphatasia have been treated with whole bone marrow or cultured osteoblasts with limited success [141–143]. In one case the patient was given MSCs 6 months after an initial bone marrow transplant with lasting improvement, possibly due to long-term engraftment. However, in each of these reports, serum ALP levels remained low despite therapeutic intervention. It is feasible that MSCs modified to express increased levels of ALP could address this shortcoming for hypophosphatasia.

OI is a congenital bone disorder that is characterized by brittle bones, primarily due to the lack of type I collagen. Current therapies are aimed at increasing bone density by antiresorptive bisphosphonate administration. Owing to their ability to differentiate into type I collagen-producing osteoblasts, MSCs have been evaluated in clinical studies of OI. Children with severe OI were treated with two infusions of allogeneic MSCs after an allogeneic bone marrow transplant. Horwitz *et al.* reported a significant increase in growth velocity of the MSC-treated children, as well as MSC engraftment in the skin, bone, and marrow stroma [144]. Chamberlain *et al.* utilized adeno-associated viral vectors to disrupt mutant COL1A1 in MSCs from OI patients and demonstrated successful deletion of the mutation. Transduced MSCs were able to produce collagen similar to wild-type cells. Furthermore, the modified MSCs were able to form ectopic bone in mice [145]. This strategy demonstrates that *ex vivo* expansion and modification of MSCs for treatment of other monogenic bone diseases may be efficacious.

Given these results, it is not surprising that combination therapies have also shown promise in musculoskeletal disease. Mice injected with RUNX2-BMP2-MSCs displayed increased bone and cartilage formation compared with BMP2-MSCs or MSCs alone [146]. BMP-VEGF-MSCs utilized in a murine model of segmental bone defects also showed enhanced bone formation compared with unmodified MSCs [147]. *In vitro* chondrogenesis was not achieved in MSCs modified to overexpress IGF-1, but gene co-modification with TGF-β or BMP2 induced greater chondrogenesis than with any of these factors alone [148,149]. This combinatorial gene modification approach demonstrated synergy between gene modifications and expanded our knowledge of the potential use of MSC-mediated gene therapy for bone and cartilage repair.

## 61.7 Gene-modified mesenchymal stromal cells in cardiovascular disease

There are over a million incidents of AMI in the USA each year. There is as yet no treatment that alleviates the permanent loss of cardiomyocytes and the ensuing fibrosis in the left ventricle that occurs after AMI. This leads to pathological left ventricular remodeling, which in turn often leads to hypokinesis of the left ventricle and death from treatment-refractory heart failure.

Many cell types have been studied for their regenerative capacity in the tissue environment after AMI. MSCs are an attractive candidate for this application and have shown promise in multiple animal models. One study reported that MSCs injected into murine hearts differentiated into cardiomyocytes and engrafted into the host tissue [150], but this has not been found in other studies. Rather, paracrine signaling from undifferentiated MSCs has been shown to promote growth and proliferation of neighboring cells. Nakanishi *et al.* found that cell-free conditioned media from MSCs promoted proliferation, inhibited apoptosis, and enhanced migration of cardiac progenitor cells *in vitro* [151]. Studies utilizing gene-modified MSCs in cardiovascular disease have aimed at increasing and optimizing the homing, survival, and engraftment of MSCs, as well as enhancing their paracrine effects, as detailed in the preclinical studies described earlier.

MSC administration has shown promise in many animal models of ischemia–reperfusion injury in disorders other than cardiovascular disease. Use of genetically modified MSCs for ischemia–reperfusion injury models in renal, limb, lung, and brain ischemia has largely focused on protecting the cells from hypoxia, as well as inducing neoangiogenesis in the damaged tissue. Our group and others have demonstrated that MSCs modified to overexpress VEGF induced endothelial cell migration *in vitro* and increased blood flow restoration in mouse and

rat models of hind limb ischemia (HLI) [32,69,70]. Similarly, another group transiently modified MSCs with VEGF polymeric nanoparticles and intramuscularly injected the cells in a mouse model of HLI. VEGF-MSC-treated mice exhibited reduced tissue fibrosis and enhanced angiogenesis after cell administration compared with animals that received unmodified MSCs [152].

Piao *et al.* utilized MSCs modified to overexpress angiopoietin-1 in a rat model of HLI and found enhanced angiogenesis as well as increased cell survival due to increased expression of AKT by the donor cells [153]. Similarly, MSCs modified with the gene for the anti-inflammatory IL-10 were used effectively to prevent ischemia–reperfusion lung injury in rats [154]. Combined, these results demonstrate that undifferentiated MSCs exhibit cytoprotective effects on other cell types, primarily through secretion of biologically active factors, and that those effects can be intensified through combinatorial strategies that promote both survival and paracrine activity of the donor MSCs.

## 61.8 Gene-modified mesenchymal stromal cells in kidney disease

Many kidney diseases are characterized by ischemic and inflammatory injury. In view of the previously described anti-inflammatory and anti-ischemia–reperfusion injury potential of MSCs, cell and gene therapies for kidney disease are being rapidly explored. Recombinant human EPO has been used successfully for many years to treat anemia in patients with end-stage renal disease [155]. Eliopoulos *et al.* examined whether subcutaneous injection of EPO-expressing MSCs could improve anemia in a mouse model of chronic renal failure and found a dose-dependent increase in hematocrit levels after subcutaneous implantation of the cells embedded in a collagen-based matrix, as well as increased cell lifespan [156]. The same group also demonstrated that co-injecting mice with renal failure with EPO-MSCs and IGF-1-MSCs led to greater hematocrit elevation as well as improved heart function [157].

Unmodified MSCs have been used to protect against acute ischemia–reperfusion injury in rat kidneys, owing to their demonstrated ability to decrease proinflammatory cytokines and increase anti-inflammatory signals in the treated kidneys [158]. Kallikrein, a potent anti-inflammatory and anti-apoptotic renoprotective molecule, has been utilized in gene-modified MSCs in a rat model of acute ischemia–reperfusion kidney injury. Kallikrein-MSCs inhibited $H_2O_2$-induced apoptosis, increased survival of proximal tubular cells *in vitro*, and led to significantly reduced renal cell apoptosis *in vivo* [159]. Yuan *et al.* observed that VEGF-MSCs co-cultured with cisplatin-treated renal epithelial cells protected the cells via antiapoptotic and mitogenic actions. Mice with cisplatin-induced renal injury showed better protection of renal function, tubular structure, and survival after VEGF-MSC administration, primarily due to improved microcirculation and cell proliferation within the damaged kidney [160].

## 61.9 Gene-modified mesenchymal stromal cells in neurological disease

MSCs promote positive effects in the brain through similar mechanisms as described earlier for other tissues. The paracrine and autocrine actions that regulate the immunomodulatory, antiapoptotic, migratory, and regenerative properties of MSCs are qualities that make them leading candidates for a cell-based approach to neurological diseases.

Many neurological diseases are characterized by, or result in, loss of neurons. There is considerable interest in utilizing MSCs for neurological diseases given the ability of MSCs to exert cytoprotective and regenerative effects through paracrine activity. Efficacy has been demonstrated in preclinical models of Alzheimer's disease, Huntington's disease (HD) (see Chapter 40), Parkinson's disease (PD), multiple sclerosis, ischemic stroke and amyotrophic lateral sclerosis (ALS) (reviewed by Tanna and Sachan [161]) [21]. MSCs can influence neural cell growth and survival, although the exact mechanisms are unclear. Crigler *et al.* found that subpopulations of human MSCs express brain-derived neurotrophic factor (BDNF) and β-nerve growth factor but not neurotrophin-3 or -4 [162]. MSCs also produce extracellular matrix molecules that support neural cell growth and attachment [163].

HD is an autosomal dominant neurodegenerative disease that is caused by variably sized polyglutamine expansion in the mutant huntingtin gene, *HTT*, leading to HTT protein-toxicity-induced death of striatal neurons and decreased expression of BDNF [164]. HD patients suffer a steady decline in emotion, motor function, and cognition before eventual death. There is currently no cure for HD. The best approach should aim to halt the progression of the disease by eliminating the mutant HTT protein and regenerating damaged neurons [165]. Our group has explored the use of MSCs as delivery vehicles for RNA inhibition, effectively decreasing expression of the mutant HTT in target cells *in vitro* [166]. The challenge with these therapies, however, is safe and sustained delivery of the therapeutic agent to the brain. MSC-based gene therapy can potentially fulfill these criteria, given their proven safety profile [29].

Canals *et al.* generated a mouse model of HD and demonstrated that disease onset is dependent on the length and levels of expressed mutant HTT, as well as the deficit in endogenous BDNF [167]. They also found that supplementation of exogenous BDNF was sufficient to restore neuronal dysfunction and extend lifespan in their R6/2 mouse model. In the YAC 128 mouse model of HD, Dey *et al.* transplanted BDNF-secreting MSCs into the striatum, significantly improving motor function and decreasing neuronal loss [168]. Existing work on the animal models of BDNF therapies for treatment of HD have been reviewed [169,170], and our team is performing studies to support a proposed future clinical trial to test safety and potential efficacy in patients with this disorder [167,168].

ALS, also known as Lou Gehrig's disease and a form of motor neurone disease, is a neurodegenerative disorder characterized

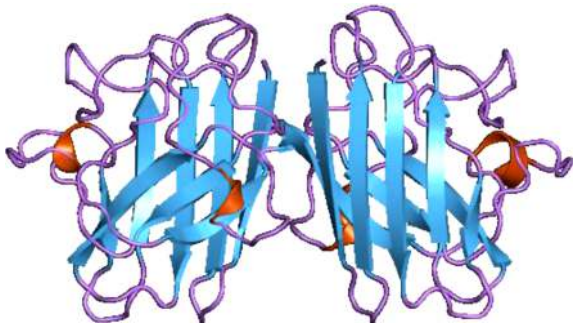


**Figure 61.8** Cartoon representation of the molecular structure of the human familial amyotrophic lateral sclerosis mutant protein G37R G37R CUZNSOD registered with 1azv code. (The structure of 1AZV is a metal-bound structure used to analyze SOD1's copper and zinc binding sites). This image, taken from Wikimedia Commons, is in the public domain because its creator, Jawahar Swaminathan and MSD staff at the *European Bioinformatics Institute*, have placed it there. (See color plate section.) *Source:* http://www.ebi.ac.uk/Information/termsofuse.html, and the courtesy link back to the creator is http://www.ebi.ac.uk/

by selective destruction of motor neurons. Failure of the neuromuscular junction in these neurons leads to paralysis and premature death, most often caused by paralysis of respiratory muscles and resulting respiratory failure [21]. Approximately 5–10% of patients with ALS have an inherited form of the disease, while the remainder are sporadic cases and with no known cause. The clinical and histopathological hallmarks, however, are identical in familial and sporadic cases [171]. The most common disease-causing mutation is a point mutation within the gene encoding $Cu^{2+}/Zn^{2+}$ superoxide dismutase 1 (SOD1).

The identification of this key mutation has led to the development of transgenic rodent models. Using SOD1 models, glial-cell-derived growth factor (GDNF) (Figure 61.8) has been shown to protect motor neurons from the typical degradation seen in untreated SOD1 mice [172]. However, clinical trials of the systemic administration of growth factor in ALS have not been successful, possibly due to their short half-life and low concentration in the target tissues.

Intramuscular delivery of adenovirus has been utilized to introduce GDNF into muscles of SOD1 mice and delay disease onset, decline in motor function, and prolong survival [172]. Suzuki *et al.* utilized MSCs engineered to express GDNF in a rat model of ALS. Animals treated with GDNF-MSCs had significantly increased neuromuscular connections, decreased motor neuron loss in the spinal cord location connected to the limb that received cells, delayed disease progression, and increased lifespan [173]. HGF, IGF-1, and VEGF have also demonstrated promising effects in rodent models [174,175]. Recently, a clinical trial utilizing autologous MSCs administered by intrathecal injection showed decreased disease progression in half of the treated patients [176]. Slowing of disease progression was linked to increased expression of trophic factors by the donor cells, demonstrating that genetic modification of MSCs to further increase the expression of these specific signals may be a promising approach for the treatment of ALS.

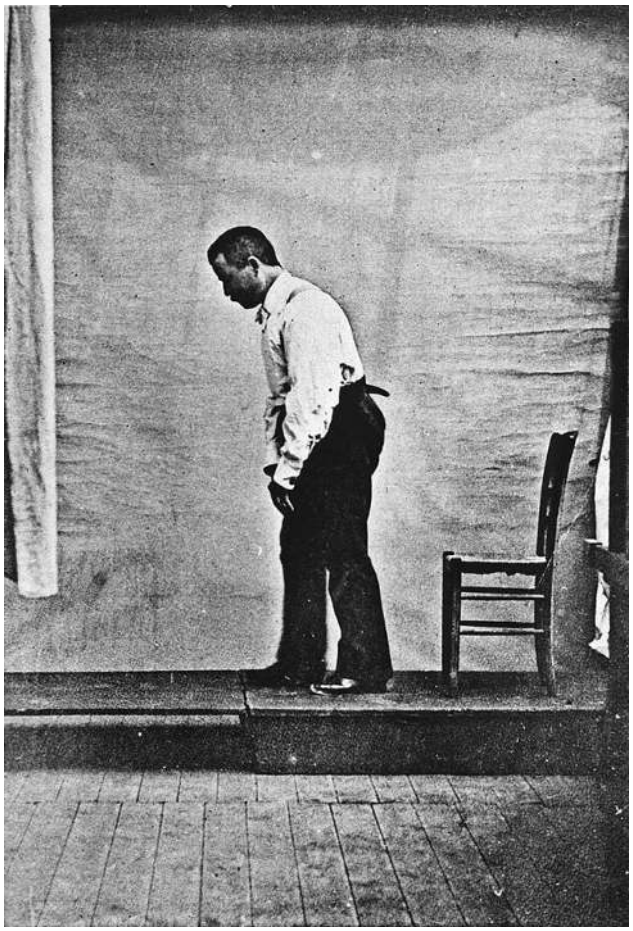


**Figure 61.9** A man with PD displaying a flexed walking posture, pictured in 1892. *Source:* nih.gov.

PD is a neurodegenerative disease of the central nervous system in which progressive loss of dopaminergic (DA) neurons in the midbrain leads to tremor and difficulty with movement including handwriting [177] (Figures 61.9 and 61.10).

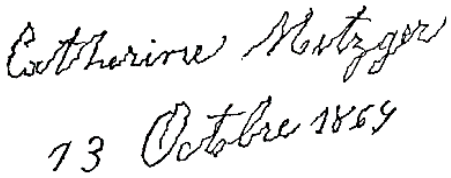



**Figure 61.10** Example of handwriting by a patient with PD possibly showing micrographia in addition to other abnormal characteristics. The original description of the text states "The strokes forming the letters are very irregular and sinuous, whilst the irregularities and sinuosities are of a very limited width. ( . . . ) the down-strokes are all, with the exception of the first letter, made with comparative firmness and are, in fact, nearly normal – the finer up-strokes, on the contrary, are all tremulous in appearance ( . . . )." *Source:* http://commons.wikimedia.org/wiki/File:Writing_by_a_Parkinson%27s_disease_patient.png#/media/File:Writing_by_a_Parkinson%27s_disease_patient.png.

Various growth factors have been examined to help recovery of damaged DA neurons (reviewed by Peterson and Nutt [178]). Specifically, GDNF has proven to be a potent neurotrophic factor for DA neurons, but, as previously discussed, sustained delivery of therapeutically relevant doses of growth factor has been a challenge. In a rat model of PD, GDNF-MSCs administered into the striatum induced regeneration of host DA striatal fibers [179]. Similar results were obtained by Somoza *et al.* using BDNF-MSCs after intrastriatal injection in the same model [180]. However, there are no current cures available for PD and, unfortunately, recent data suggest that the disease can be propagated to engrafted donor neurons [181].

## 61.10 Gene-modified mesenchymal stromal cells in other nonmalignant diseases

### 61.10.1 Hemophilia

Hemophilia is an X-linked genetic disorder in which patients have abnormal clotting due to lack of specific coagulation factors. Hemophilia A is due to a deficiency of coagulation factor VIII (FVIII). MSCs genetically modified to express FVIII (FVIII-MSCs) have been shown to correct the hemophilia A phenotype in affected mouse models [182]. The same finding was made using supernatant from FVIII-MSC cultures. Hemophilia B patients are deficient in FIX. Dodd *et al.* generated FIX-MSCs with sustained expression of functional FIX before and after differentiation into adipogenic, osteoblastic, and chondrogenic cells *in vitro* [183]. Intravenous delivery of naked FIX plasmid into hemophilia B mice induced therapeutic levels of human FIX for over a year, but acute hepatic damage was also observed [25]. The use of FIX-MSCs for safe and sustained delivery of clotting factor may be a successful long-term treatment of hemophilia B.

### 61.10.2 Metachromatic leukodystrophy

Metachromatic leukodystrophy is a genetic lysosomal storage disease that affects nerves, muscles, and other organs with increased severity over time. In 2002 Koç *et al.* treated patients with metachromatic leukodystrophy with MSCs after they had received an HLA-matched bone marrow transplant. Four out of six patients showed increased improvement in nerve conduction velocities and bone mineral density after MSC infusion. However, there were no changes in the patients' overall health or development. Despite the lack of reversal of disease pathology, they found that donor MSCs were still detectable 60 days after administration in two patients and concluded that allogeneic MSC infusion was safe [184].

### 61.10.3 Mucopolysaccharidosis type VII

Mucopolysaccharidosis type VII (MPSVII), another inherited lysosomal storage disease, characterized by deficiency of the β-glucuronidase (GUSB) enzyme, has also been explored as a candidate for MSC-mediated gene therapy. Meyerrose *et al.* generated human GUSB-MSCs to transplant into immune-deficient MPSVII mice. They observed increased circulating GUSB levels, normalized levels of lysosomal enzymes, and improved retinal function. They also observed normal trafficking of the gene-modified cells and long-term persistence of GUSB-MSCs delivering therapeutic levels of protein [32].

### 61.10.4 Diabetes mellitus

MSCs gene modified to express pancreatic duodenal homeobox-1 (PDX-1) have been generated with the goal of producing functional insulin-secreting cells to treat diabetes mellitus. PDX-1-MSCs were implanted in a hyperglycemic rat model and reduced blood glucose levels to normal [185]. Li *et al.* were able to obtain euglycemia in streptozotocin-induced diabetic mice treated with PDX-1-MSCs for 42 days after administration [186].

## 61.11 Conclusions and future directions

There is enormous potential of the use of gene-modified MSCs for delivery of enzymes and growth factors or to increase supplementation of therapeutically beneficial signals. A summary of these is shown in Figure 61.11.

Given the substantial safety concerns with gene therapies, as exemplified by gene therapy clinical trials of the 1990s that led to the deaths of several patients and temporarily halted gene therapy trials [23], other approaches for growth factor supplementation have been explored. Unfortunately, direct administration of growth factors or naked viral plasmids carries additional caveats. Concerns with direct administration of growth factors include expense, difficulty in delivering them in a sustained manner due to their instability, and the requirement for a large quantity of them to produce effects at the site of tissue damage. Similar concerns exist with direct plasmid supplementation with further safety concerns.

Gene-modified MSCs represent an approach that may resolve many of the problems. Unmodified MSCs can be administered to a site of injury, engraft, and/or respond to endogenous signals to induce secretion of paracrine signals required for repair. Furthermore, MSCs are part of an innate tissue healing response and are able to migrate to sites of tissue injury and hypoxia in response to cell trafficking signals [37,38,187]. MSCs possess immunomodulatory functions that further contribute to a pro-regenerative microenvironment and are able to evade immune detection by the host. By modifying the genes responsible for these endogenous functions, or engineering the cells to express a therapeutic protein for exogenous supplementation, we can utilize MSCs to their greatest potential as powerful delivery vehicles and protein synthesis factories.

Safety concerns are at the forefront of preclinical and clinical study development. Ongoing studies seek to optimize *in vitro* culture conditions to ensure development of a safe cell product and clinically acceptable expansion protocols. Xeno-free culture conditions are being explored to minimize the risk of immune

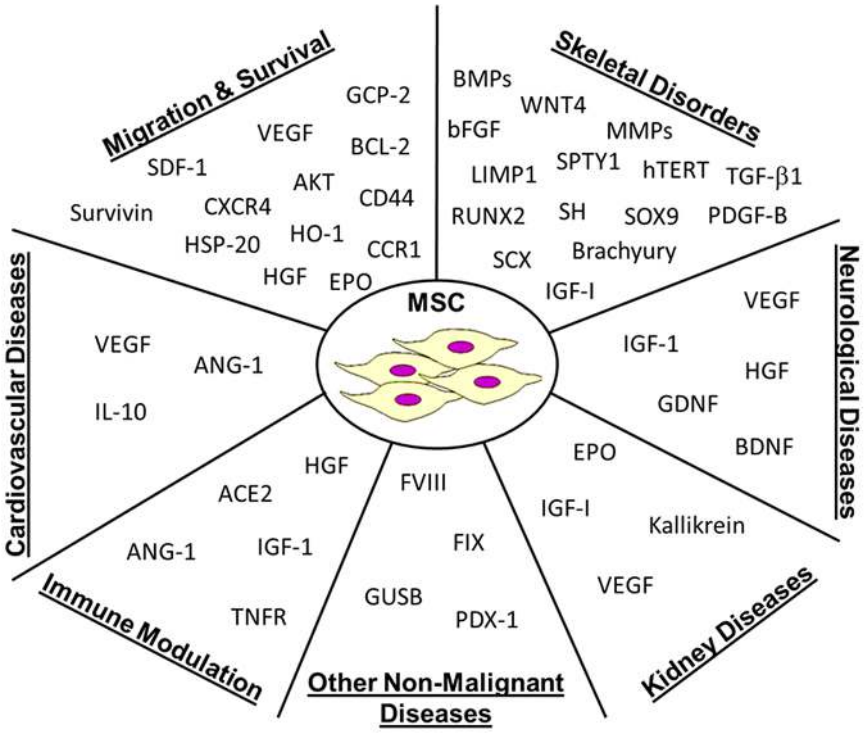



**Figure 61.11** A summary of targets for genetic modification of MSCs and their therapeutic applications.

reactions and pathogen transmission [188]. While there has been no documented evidence of malignant transformation of human MSCs, it remains critical to maintain standard operating procedures and *in vitro* culture protocols and to employ sensitive karyotyping methods. In some instances it may be preferable to develop methods to induce beneficial effects without gene modification of the cells. For example, exposure to hypoxic culture conditions can also promote expression of the survival and proangiogenic signals in MSCs (reviewed by Haque *et al.* [189]. and Beegle *et al.* [190].).

The success of future MSC-mediated gene therapy trials depends on the development of safe standard operating procedures and protocols for the expansion and genetic engineering of MSCs. Suicide genes, such as the herpes simplex virus type 1–thymidine kinase system, have been utilized to circumvent concern over serious side effects from gene-modified cells [191,192]. However, this approach must be considered with caution, as suicide-gene-induced cell death can have bystander effects on surrounding cells, and MSCs are known to transfer proteins from cell to cell.

It is also important to follow cell fate after injection in order to understand and resolve any potential adverse events. Nontherapeutic tracking genes such as eGFP and luciferase are prohibited for use in clinical trials. Currently, the only way to interrogate cell fate after injection is through the use of these tracking genes in animal models, with translation into larger animal models once efficacy has been demonstrated.

MSCs have demonstrated safety and efficacy in a number of clinical trials in cardiovascular diseases, neurological diseases, and GVHD, but issues with efficacy and reproducibility remain [193–197]. Additionally, both the optimal dose of gene-modified MSCs and the optimal delivery route need to be established, and this will vary depending on the tissue or organ to be healed or repaired. The route of administration can have significant impacts on the efficacy of treatment. Kashiwakura *et al.* found that intra-articular injection of FVIII-MSCs was significantly better at alleviating hemarthrosis and hemophilic arthropathy in a mouse model of hemophilia A compared with intravenous and subcutaneous administration [182]. Timing and dose of administration of gene-modified MSCs can also impact therapeutic efficacy. Richardson *et al.* tested low and high doses of MSCs immediately or 7 days after initiation of AMI in rats and found that both the cell doses given immediately after AMI demonstrated efficacy, with the high dose producing the greatest increase in left ventricular ejection fraction and the greatest decrease in pathological remodeling of the left ventricle [198]. Interestingly, they found that later cell administration benefited the noninfarct region, but did not significantly contribute to healing in the infarct region. Fiore *et al.* found in a mouse model of liver cirrhosis that repeated injections of IGF-1-MSCs yielded the greatest antifibrinogenic effects [99].

The cell delivery vehicle can also be important in MSC-based gene therapies. Sayyar *et al.* tested the cell-matrix interactions between FIX-MSC in an RGD-alginate scaffold compared with encapsulation within fibrinogen-alginate microcapsules, and found that FIX-MSCs encapsulated in fibrinogen-alginate microcapsules showed increased FIX expression while FIX-MSCs in RGD-alginate scaffolds showed enhanced cell attachment and

viability of the cells [199]. This research indicated RDG-alginate scaffold delivery may be preferable for applications that require cell attachment and viability over enhanced gene expression.

Finally, the tissue source of donor MSCs may be a critical factor for cell and gene therapy. Park *et al.* examined the chondrogenic potential of MSCs isolated from bone marrow, adipose tissue, and perichondrium/periosteum for articular cartilage repair and found that perichondrium/periosteum- and bone-marrow-derived MSCs were superior to adipose-tissue-derived MSCs, even though cells from all three sources required BMP2 for initiation and support of chondrogenesis. Many of these factors are not sufficiently optimized when designing MSC-based clinical trials and may be a potential explanation for lack of repeatable efficacy. There is a need to identify a standard tissue donor and source, expansion procedures, specific defining markers, dosing, and administration strategies.

## References


1 Pittenger, M. F., Mackay, A. M., Beck, S. C., *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science (New York, N.Y.)* (1999)**284**,143–147.

2 Caplan, A. I. and Dennis, J. E. Mesenchymal stem cells as trophic mediators. *Journal of Cellular Biochemistry* (2006) **98**,1076–1084.

3 Phinney, D. G. and Prockop, D. J. Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair – current views. *Stem Cells* (2007) **25**,2896–2902.

4 Meyerrose, T., Olson, S., Pontow, S., *et al.* Mesenchymal stem cells for the sustained *in vivo* delivery of bioactive factors. *Advanced Drug Delivery Reviews* (2010)**62**,1167–1174.

5 Horwitz, E. M., Prockop, D. J., Fitzpatrick, L. A., *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nature Medicine* (1999)**5**,309–313.

6 Le Blanc, K., Frassoni, F., Ball, L., *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* (2008)**371**,1579–1586.

7 Parekkadan, B. and Milwid, J. M. Mesenchymal stem cells as therapeutics. *Annual Review of Biomedical Engineering* (2010) **12**,87–117.

8 Friedenstein, A. J., Chailakhjan, R. K., and Lalykina, K. S. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell and Tissue Kinetics* (1970) **3**,393–403.

9 Da Silva Meirelles, L., Chagastelles, P. C., and Nardi, N. B. Mesenchymal stem cells reside in virtually all post-natal organs and tissues. *Journal of Cell Science* (2006)**119**,2204–2213.

10 Lee, O. K., Kuo, T. K., Chen, W. M., *et al.* Isolation of multipotent mesenchymal stem cells from umbilical cord blood. *Blood* (2004) **103**,1669–1675.

11 Crisan, M., Chen, C. W., Corselli, M., *et al.* Perivascular multipotent progenitor cells in human organs. *Annals of the New York Academy of Sciences* (2009)**1176**,118–123.

12 Gotherstrom, C., West, A., Liden, J., *et al.* Difference in gene expression between human fetal liver and adult bone marrow mesenchymal stem cells. *Haematologica* (2005)**90**,1017–1026.

13 In 't Anker, P. S., Noort, W. A., Scherjon, S. A., *et al.* Mesenchymal stem cells in human second-trimester bone marrow, liver, lung, and spleen exhibit a similar immunophenotype but a heterogeneous multilineage differentiation potential. *Haematologica* (2003) **88**,845–852.

14 Kern, S., Eichler, H., Stoeve, J., *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* (2006)**24**,1294–1301.

15 Tokcaer-Keskin, Z., Akar, A. R., Ayaloglu-Butun, F., *et al.* Timing of induction of cardiomyocyte differentiation for *in vitro* cultured mesenchymal stem cells: a perspective for emergencies. *Canadian Journal of Physiology and Pharmacology* (2009)**87**,143–150.

16 Dezawa, M., Kanno, H., Hoshino, M., *et al.* Specific induction of neuronal cells from bone marrow stromal cells and application for autologous transplantation. *The Journal of Clinical Investigation* (2004)**113**,1701–1710.

17 Luk, J. M., Wang, P. P., Lee, C. K., *et al.* Hepatic potential of bone marrow stromal cells: development of *in vitro* co-culture and intraportal transplantation models. *Journal of Immunological Methods* (2005)**305**,39–47.

18 Dominici, M., Le Blanc, K., Mueller, I., *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. *The International Society for Cellular Therapy position statement. Cytotherapy* (2006) **8**,315–317.

19 Lazarus, H. M., Haynesworth, S. E., Gerson, S. L., *et al. Ex vivo* expansion and subsequent infusion of human bone marrow-derived stromal progenitor cells (mesenchymal progenitor cells): implications for therapeutic use. *Bone Marrow Transplantation* (1995)**16**,557–564.

20 Lalu, M. M., McIntyre, L., Pugliese, C., *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* (2012)**7**,e47559.

21 Joyce, N., Annett, G., Wirthlin, L., *et al.* Mesenchymal stem cells for the treatment of neurodegenerative disease. *Regenerative Medicine* (2010)**5**,933–946.

22 Salem, H. K. and Thiemermann, C. Mesenchymal stromal cells: current understanding and clinical status. *Stem Cells* (2010) **28**,585–596.

23 Raper, S. E., Chirmule, N., Lee, F. S., *et al.* Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Molecular Genetics and Metabolism* (2003)**80**,148–158.

24 Govender, S., Csimma, C., Genant, H. K., *et al.* Recombinant human bone morphogenetic protein-2 for treatment of open tibial fractures: a prospective, controlled, randomized study of four hundred and fifty patients. *The Journal of Bone and Joint Surgery. American Volume* (2002)**84-A**,2123–2134.

25 Ye, X., Loeb, K. R., Stafford, D. W., *et al.* Complete and sustained phenotypic correction of hemophilia B in mice following hepatic gene transfer of a high-expressing human factor IX plasmid. *Journal of Thrombosis and Haemostasis* (2003)**1**,103–111.

26 Montini, E., Cesana, D., Schmidt, M., *et al.* Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. *Nature Biotechnology* (2006)**24**,687–696.

27 Koegel, H., von Tobel, L., Schäfer, M., *et al.* Loss of serum response factor in keratinocytes results in hyperproliferative skin disease in mice. *The Journal of Clinical Investigation* (2009)**119**,899–910.

28 Aiuti, A. and Roncarolo, M. G. Ten years of gene therapy for primary immune deficiencies. *Hematology/The Education Program of the American Society of Hematology. American Society of Hematology. Education Program* (2009),682–689.

29 Bauer, G., Dao, M. A., Case, S. S., *et al. In vivo* biosafety model to assess the risk of adverse events from retroviral and lentiviral vectors. *Molecular Therapy* (2008)**16**,1308–1315.

30 Waehler, R., Russell, S. J., and Curiel, D. T. Engineering targeted viral vectors for gene therapy. *Nature Reviews. Genetics* (2007) **8**,573–587.

31 Jung, Y., Bauer, G., and Nolta, J. A. Concise review: induced pluripotent stem cell-derived mesenchymal stem cells: progress toward safe clinical products. *Stem Cells* (2012)**30**,42–47.

32 Meyerrose, T. E., Roberts, M., Ohlemiller, K. K., *et al.* Lentiviral-transduced human mesenchymal stem cells persistently express therapeutic levels of enzyme in a xenotransplantation model of human disease. *Stem Cells* (2008)**26**,1713–1722.

33 Stephenson, J. Studies illuminate cause of fatal reaction in gene-therapy trial. *JAMA* (2001)**285**,2570.

34 Francois, S., Bensidhoum, M., Mouiseddine, M., *et al.* Local irradiation not only induces homing of human mesenchymal stem cells at exposed sites but promotes their widespread engraftment to multiple organs: a study of their quantitative distribution after irradiation damage. *Stem Cells* (2006)**24**,1020–1029.

35 Rochefort, G. Y., Delorme, B., Lopez, A., *et al.* Multipotential mesenchymal stem cells are mobilized into peripheral blood by hypoxia. *Stem Cells* (2006)**24**,2202–2208.

36 Granero-Molto, F., Weis, J. A., Longobardi, L., and Spagnoli A. Role of mesenchymal stem cells in regenerative medicine: application to bone and cartilage repair. *Expert Opinion on Biological Therapy* (2008)**8**,255–268.

37 Karp, J. M. and Leng Teo, G. S. Mesenchymal stem cell homing: the devil is in the details. *Cell Stem Cell* (2009)**4**,206–216.

38 Meyerrose, T. E., De Ugarte, D. A., Hofling, A. A., *et al. In vivo* distribution of human adipose-derived mesenchymal stem cells in novel xenotransplantation models. *Stem Cells* (2007)**25**,220–227.

39 Barbash, I. M., Chouraqui, P., Baron, J., *et al.* Systemic delivery of bone marrow-derived mesenchymal stem cells to the infarcted myocardium: feasibility, cell migration, and body distribution. *Circulation* (2003)**108**,863–868.

40 Sasaki, M., Abe, R., Fujita, Y., *et al.* Mesenchymal stem cells are recruited into wounded skin and contribute to wound repair by transdifferentiation into multiple skin cell type. *Journal of Immunology* (2008)**180**,2581–2587.

41 Abbott, J. D., Huang, Y., Liu, D., *et al.* Stromal cell-derived factor-1α plays a critical role in stem cell recruitment to the heart after myocardial infarction but is not sufficient to induce homing in the absence of injury. *Circulation* (2004)**110**,3300–3305.

42 Hiasa, K., Ishibashi, M., Ohtani, K., *et al.* Gene transfer of stromal cell-derived factor-1α enhances ischemic vasculogenesis and angiogenesis via vascular endothelial growth factor/endothelial nitric oxide synthase-related pathway: next-generation chemokine therapy for therapeutic neovascularization. *Circulation* (2004)**109**,2454–2461.

43 Rombouts, W. J. and Ploemacher, R. E. Primary murine MSC show highly efficient homing to the bone marrow but lose homing ability following culture. *Leukemia* (2003)**17**,160–170.

44 Wynn, R. F., Hart, C. A., Corradi-Perini, C., *et al.* A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. *Blood* (2004)**104**,2643–2645.

45 Hung, S. C., Pochampally, R. R., Hsu, S. C., *et al.* Short-term exposure of multipotent stromal cells to low oxygen increases their expression of CX3CR1 and CXCR4 and their engraftment *in vivo*. *PLoS ONE* (2007)**2**,e416.

46 Cheng, Z., Ou, L., Zhou, X., *et al.* Targeted migration of mesenchymal stem cells modified with CXCR4 gene to infarcted myocardium improves cardiac performance. *Molecular Therapy* (2008) **16**,571–579.

47 Zhang, D., Fan, G. C., Zhou, X., *et al.* Over-expression of CXCR4 on mesenchymal stem cells augments myoangiogenesis in the infarcted myocardium. *Journal of Molecular and Cellular Cardiology* (2008)**44**,281–292.

48 Ma, H. C., Shi, X. L., Ren, H. Z., *et al.* Targeted migration of mesenchymal stem cells modified with CXCR4 to acute failing liver improves liver regeneration. *World Journal of Gastroenterology* (2014)**20**,14884–14894.

49 Wang, Z., Wang, Y., Wang, Z., *et al.* Engineered mesenchymal stem cells with enhanced tropism and paracrine secretion of cytokines and growth factors to treat traumatic brain injury. *Stem Cells* (2015) **33**,456–467.

50 Ho, C. Y., Sanghani, A., Hua, J., *et al.* Mesenchymal stem cells with increased stromal cell-derived factor 1 expression enhanced fracture healing. *Tissue Engineering. Part A* (2015)**21**(3–4),594–602.

51 Sackstein, R., Merzaban, J. S., Cain, D. W., *et al. Ex vivo* glycan engineering of CD44 programs human multipotent mesenchymal stromal cell trafficking to bone. *Nature Medicine* (2008) **14**,181–187.

52 Huang, J., Zhang, Z., Guo, J., *et al.* Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. *Circulation Research* (2010)**106**,1753–1762.

53 Kim, S. W., Lee, D. W., Yu, L. H., *et al.* Mesenchymal stem cells overexpressing GCP-2 improve heart function through enhanced angiogenic properties in a myocardial infarction model. *Cardiovascular Research* (2012)**95**,495–506.

54 Liu, X., Pachori, A. S., Ward, C. A., *et al.* Heme oxygenase-1 (HO-1) inhibits postmyocardial infarct remodeling and restores ventricular function. *FASEB Journal* (2006)**20**,207–216.

55 Zeng, B., Chen, H., Zhu, C., *et al.* Effects of combined mesenchymal stem cells and heme oxygenase-1 therapy on cardiac performance. *European Journal of Cardio-thoracic Surgery* (2008)**34**,850–856.

56 Jiang, Y. B., Zhang, X. L., Tang, Y. L., *et al.* Effects of heme oxygenase-1 gene modulated mesenchymal stem cells on vasculogenesis in ischemic swine hearts. *Chinese Medical Journal* (2011) **124**,401–407.

57 Tsubokawa, T., Yagi, K., Nakanishi, C., *et al.* Impact of anti-apoptotic and anti-oxidative effects of bone marrow mesenchymal stem cells with transient overexpression of heme oxygenase-1 on myocardial ischemia. *American Journal of Physiology. Heart and Circulatory Physiology* (2010)**298**,H1320–H1329.

58 Zeng, B., Lin, G., Ren, X., *et al.* Over-expression of HO-1 on mesenchymal stem cells promotes angiogenesis and improves myocardial function in infarcted myocardium. *Journal of Biomedical Science* (2010)**17**,80.

59 Tang, Y. L., Tang, Y., Zhang, Y. C., *et al.* Improved graft mesenchymal stem cell survival in ischemic heart with a hypoxia-regulated heme oxygenase-1 vector. *Journal of the American College of Cardiology* (2005)**46**,1339–1350.

60 Li, W., Ma, N., Ong, L. L., *et al.* Bcl-2 engineered MSCs inhibited apoptosis and improved heart function. *Stem Cells* (2007) **25**,2118–2127.

61 Copland, I. B., Jolicoeur, E. M., Gillis, M. A., *et al.* Coupling erythropoietin secretion to mesenchymal stromal cells enhances their regenerative properties. *Cardiovascular Research* (2008) **79**,405–415.

62 Fan, L., Lin, C., Zhuo, S., *et al.* Transplantation with survivin-engineered mesenchymal stem cells results in better prognosis in a rat model of myocardial infarction. *European Journal of Heart Failure* (2009)**11**,1023–1030.

63 Wang, X., Zhao, T., Huang, W., *et al.* Hsp20-engineered mesenchymal stem cells are resistant to oxidative stress via enhanced activation of Akt and increased secretion of growth factors. *Stem Cells* (2009)**27**,3021–3031.

64 Wang, D., Shen, W., Zhang, F., *et al.* Connexin43 promotes survival of mesenchymal stem cells in ischaemic heart. *Cell Biology International* (2010)**34**,415–423.

65 Datta, S. R., Brunet, A., and Greenberg, M. E. Cellular survival: a play in three Akts. *Genes & Development* (1999)**13**,2905–2927.

66 Mangi, A. A., Noiseux, N., Kong, D., *et al.* Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nature Medicine* (2003)**9**,1195–1201.

67 Yu, Y. S., Shen, Z. Y., Ye, W. X., *et al.* AKT-modified autologous intracoronary mesenchymal stem cells prevent remodeling and repair in swine infarcted myocardium. *Chinese Medical Journal* (2010)**123**,1702–1708.

68 Gnecchi, M., He, H., Liang, O. D., *et al.* Paracrine action accounts for marked protection of ischemic heart by Akt-modified mesenchymal stem cells. *Nature Medicine* (2005)**11**,367–368.

69 Wang, Y., Haider, H. K., Ahmad, N., *et al.* Combining pharmacological mobilization with intramyocardial delivery of bone marrow cells over-expressing VEGF is more effective for cardiac repair. *Journal of Molecular and Cellular Cardiology* (2006)**40**,736–745.

70 Gao, F., He, T., Wang, H., *et al.* A promising strategy for the treatment of ischemic heart disease: mesenchymal stem cell-mediated vascular endothelial growth factor gene transfer in rats. *The Canadian Journal of Cardiology* (2007)**23**,891–898.

71 Matsumoto, R., Omura, T., Yoshiyama, M., *et al.* Vascular endothelial growth factor-expressing mesenchymal stem cell transplantation for the treatment of acute myocardial infarction. *Arteriosclerosis, Thrombosis, and Vascular Biology* (2005)**25**,1168–1173.

72 Deuse, T., Peter, C., Fedak, P. W., *et al.* Hepatocyte growth factor or vascular endothelial growth factor gene transfer maximizes mesenchymal stem cell-based myocardial salvage after acute myocardial infarction. *Circulation* (2009)**120**,S247–S254.

73 Duan, H. F., Wu, C. T., Wu, D. L., *et al.* Treatment of myocardial ischemia with bone marrow-derived mesenchymal stem cells overexpressing hepatocyte growth factor. *Molecular Therapy* (2003) **8**,467–474.

74 Guo, Y., He, J., Wu, J., *et al.* Locally overexpressing hepatocyte growth factor prevents post-ischemic heart failure by inhibition of apoptosis via calcineurin-mediated pathway and angiogenesis. *Archives of Medical Research* (2008)**39**,179–188.

75 Guo, Y. H., He, J. G., Wu, J. L., *et al.* Hepatocyte growth factor and granulocyte colony-stimulating factor form a combined neovasculogenic therapy for ischemic cardiomyopathy. *Cytotherapy* (2008) **10**,857–867.

76 Pontikoglou, C., Deschaseaux, F., Sensebé, L., and Papadaki, H. A. Bone marrow mesenchymal stem cells: biological properties and their role in hematopoiesis and hematopoietic stem cell transplantation. *Stem Cell Reviews* (2011)**7**,569–589.

77 Tse, W. T., Pendleton, J. D., Beyer, W. M., *et al.* Suppression of allogeneic T-cell proliferation by human marrow stromal cells: implications in transplantation. *Transplantation* (2003)**75**,389–397.

78 Le Blanc, K. Mesenchymal stromal cells: tissue repair and immune modulation. *Cytotherapy* (2006)**8**,559–561.

79 Ishiwata, I., Yasuda, M., Hirano, T., and Ishikawa, H. Establishment and characterization of HEPFT, a cell line derived from hepatoid carcinoma of the fallopian tube, with special reference to α-fetoprotein, lectin affinity and histogenesis. *Human Cell* (2007) **20**,119–130.

80 Vetyska, J. and Krusina, L. Indications for peroperative cholangiography in cholecystectomy. *Rozhledy v Chirurgii: Mesicnik Ceskoslovenske Chirurgicke Spolecnosti* (1990)**69**,605–610 (in Czech).

81 Aggarwal, S. and Pittenger, M. F. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* (2005) **105**,1815–1822.

82 Sato, K., Ozaki, K., Oh, I., *et al.* Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells. *Blood* (2007)**109**,228–234.

83 Di Nicola, M., Carlo-Stella, C., Magni, M., *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* (2002) **99**,3838–3843.

84 Groh, M. E., Maitra, B., Szekely, E., and Koç, O. N. Human mesenchymal stem cells require monocyte-mediated activation to suppress alloreactive T cells. *Experimental Hematology* (2005) **33**,928–934.

85 Meisel, R., Zibert, A., Laryea, M., *et al.* Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine 2,3-dioxygenase-mediated tryptophan degradation. *Blood* (2004) **103**,4619–4621.

86 Granero-Moltó, F., Weis, J. A., Miga, M. I., *et al.* Regenerative effects of transplanted mesenchymal stem cells in fracture healing. *Stem Cells* (2009)**27**,1887–1898.

87 Crop, M., Baan, C., Weimar, W., and Hoogduijn, M. Potential of mesenchymal stem cells as immune therapy in solid-organ transplantation. *Transplant International* (2009)**22**,365–376.

88 Crop, M. J., Baan, C. C., Korevaar, S. S., *et al.* Donor-derived mesenchymal stem cells suppress alloreactivity of kidney transplant patients. *Transplantation* (2009)**87**,896–906.

89 Bartholomew, A., Sturgeon, C., Siatskas, M., *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Experimental Hematology* (2002) **30**,42–48.

90 Ge, W., Jiang, J., Baroja, M. L., *et al.* Infusion of mesenchymal stem cells and rapamycin synergize to attenuate alloimmune responses and promote cardiac allograft tolerance. *American Journal of Transplantation* (2009)**9**,1760–1772.

91 Bacigalupo, A. Management of acute graft-versus-host disease. *British Journal of Haematology* (2007)**137**,87–98.

92 Le Blanc, K., Rasmusson, I., Sundberg, B., *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* (2004)**363**,1439–1441.

93 Ball, L. M., Bernardo, M. E., Roelofs, H., *et al.* Cotransplantation of ex vivo expanded mesenchymal stem cells accelerates lymphocyte recovery and may reduce the risk of graft failure in haploidentical hematopoietic stem-cell transplantation. *Blood* (2007)**110**,2764–2767.

94 Ringdén, O., Uzunel, M., Rasmusson, I., *et al.* Mesenchymal stem cells for treatment of therapy-resistant graft-versus-host disease. *Transplantation* (2006)**81**,1390–1397.

95 He, H. L., Liu, L., Chen, Q. H., *et al.* MSCs modified with ACE2 restore endothelial function following LPS challenge by inhibiting the activation of RAS. *Journal of Cellular Physiology* (2015) **230**,691–701.

96 Xu, J., Qu, J., Cao, L., *et al.* Mesenchymal stem cell-based angiopoietin-1 gene therapy for acute lung injury induced by lipopolysaccharide in mice. *The Journal of Pathology* (2008) **214**,472–481.

97 Kubota, T., Bounoutas, G. S., Miyagishima, M., *et al.* Soluble tumor necrosis factor receptor abrogates myocardial inflammation but not hypertrophy in cytokine-induced cardiomyopathy. *Circulation* (2000)**101**,2518–2525.

98 Bao, C., Guo, J., Zheng, M., *et al.* Enhancement of the survival of engrafted mesenchymal stem cells in the ischemic heart by TNFR gene transfection. *Biochemistry and Cell Biology* (2010) **88** (4),629–634.

99 Fiore, E. J., Bayo, J., Garcia, M. G., *et al.* Mesenchymal stromal cells engineered to produce IGF-I by recombinant adenovirus ameliorate liver fibrosis in mice. *Stem Cells and Development* (2015) **24**,791–801.

100 Zhu, J. and Chen, Y. Effect of human hepatocyte growth factor gene-modified bone marrow mesenchymal stem cells on immunological rejection after allograft liver transplantation in rats. *Zhongguo Xiu Fu Chong Jian Wai Ke Za Zhi* (2011)**25**,871–876 (in Chinese).

101 Pacini, S., Spinabella, S., Trombi, L., *et al.* Suspension of bone marrow-derived undifferentiated mesenchymal stromal cells for repair of superficial digital flexor tendon in race horses. *Tissue Engineering* (2007)**13**,2949–2955.

102 Hogan, B. L. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. *Genes & Development* (1996) **10**,1580–1594.

103 Oreffo, R. O. Growth factors for skeletal reconstruction and fracture repair. *Current Opinion in Investigational Drugs* (2004) **5**,419–423.

104 Tsuji, K., Bandyopadhyay, A., Harfe, B. D., *et al.* BMP2 activity, although dispensable for bone formation, is required for the initiation of fracture healing. *Nature Genetics* (2006) **38**,1424–1429.

105 McKay, W. F., Peckham, S. M., and Badura, J. M. A comprehensive clinical review of recombinant human bone morphogenetic protein-2 (INFUSE Bone Graft). *International Orthopaedics* (2007) **31**,729–734.

106 Sugiyama, O., An, D. S., Kung, S. P., *et al.* Lentivirus-mediated gene transfer induces long-term transgene expression of BMP-2 *in vitro* and new bone formation *in vivo*. *Molecular Therapy* (2005) **11**,390–398.

107 Turgeman, G., Pittman, D. D., Müller, R., *et al.* Engineered human mesenchymal stem cells: a novel platform for skeletal cell mediated gene therapy. *The Journal of Gene Medicine* (2001)**3**,240–251.

108 Aslan, H., Zilberman, Y., Arbeli, V., *et al.* Nucleofection-based *ex vivo* nonviral gene delivery to human stem cells as a platform for tissue regeneration. *Tissue Engineering* (2006)**12**,877–889.

109 Myers, T. J., Granero-Molto, F., Longobardi, L., *et al.* Mesenchymal stem cells at the intersection of cell and gene therapy. *Expert Opinion on Biological Therapy* (2010)**10**,1663–1679.

110 Zachos, T., Diggs, A., Weisbrode, S., *et al.* Mesenchymal stem cell-mediated gene delivery of bone morphogenetic protein-2 in an articular fracture model. *Molecular Therapy* (2007) **15**,1543–1550.

111 Krebsbach, P. H., Gu, K., Franceschi, R. T., and Rutherford, R. B. Gene therapy-directed osteogenesis: BMP-7-transduced human fibroblasts form bone *in vivo*. *Human Gene Therapy* (2000) **11**,1201–1210.

112 Zhang, X. S., Linkhart, T. A., Chen, S. T., *et al.* Local *ex vivo* gene therapy with bone marrow stromal cells expressing human BMP4 promotes endosteal bone formation in mice. *The Journal of Gene Medicine* (2004)**6**,4–15.

113 Breitbart, A. S., Grande, D. A., Mason, J. M., *et al.* Gene-enhanced tissue engineering: applications for bone healing using cultured periosteal cells transduced retrovirally with the BMP-7 gene. *Annals of Plastic Surgery* (1999)**42**,488–495.

114 Hidaka, C., Quitoriano, M., Warren, R. F., and Crystal, R. G. Enhanced matrix synthesis and in vitro formation of cartilage-like tissue by genetically modified chondrocytes expressing BMP-7. *Journal of Orthopaedic Research* (2001)**19**,751–758.

115 Wang, Q. W., Chen, Z. L., and Piao, Y. J. Mesenchymal stem cells differentiate into tenocytes by bone morphogenetic protein (BMP) 12 gene transfer. *Journal of Bioscience and Bioengineering* (2005) **100**,418–422.

116 Park, J., Gelse, K., Frank, S., *et al.* Transgene-activated mesenchymal cells for articular cartilage repair: a comparison of primary bone marrow-, perichondrium/periosteum- and fat-derived cells. *The Journal of Gene Medicine* (2006)**8**,112–125.

117 Edwards, P. C., Ruggiero, S., Fantasia, J., *et al.* Sonic hedgehog gene-enhanced tissue engineering for bone regeneration. *Gene Therapy* (2005)**12**,75–86.

118 Guo, X., Zheng, Q., Kulbatski, I., *et al.* Bone regeneration with active angiogenesis by basic fibroblast growth factor gene transfected mesenchymal stem cells seeded on porous β-TCP ceramic scaffolds. *Biomedical Matererials* (2006)**1**,93–99.

119 Chang, J., Sonoyama, W., Wang, Z., *et al.* Noncanonical Wnt-4 signaling enhances bone regeneration of mesenchymal stem cells in craniofacial defects through activation of p38 MAPK. *The Journal of Biological Chemistry* (2007)**282**,30938–30948.

120 Pola, E., Gao, W., Zhou, Y., *et al.* Efficient bone formation by gene transfer of human LIM mineralization protein-3. *Gene Therapy* (2004)**11**,683–693.

121 Viggeswarapu, M., Boden, S. D., Liu, Y., *et al.* Adenoviral delivery of LIM mineralization protein-1 induces new-bone formation *in vitro* and *in vivo*. *The Journal of Bone and joint Surgery. American Volume* (2001)**83-A**,364–376.

122 Granero-Molto, F., Myers, T. J., Weis, J. A., *et al.* Mesenchymal stem cells expressing insulin-like growth factor-I (MSCIGF) promote fracture healing and restore new bone formation in Irs1 knockout

mice: analyses of MSCIGF autocrine and paracrine regenerative effects. *Stem Cells* (2011)**29**,1537–1548.

123 Goodrich, L. R., Hidaka, C., Robbins, P. D., *et al.* Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. *The Journal of Bone and Joint Surgery. British Volume* (2007)**89**,672–685.

124 Cucchiarini, M., Madry, H., Ma, C., *et al.* Improved tissue repair in articular cartilage defects in vivo by rAAV-mediated overexpression of human fibroblast growth factor 2. *Molecular Therapy* (2005) **12**,229–238.

125 Cucchiarini, M. and Madry, H. Overexpression of human IGF-I via direct rAAV-mediated gene transfer improves the early repair of articular cartilage defects *in vivo*. *Gene Therapy* (2014) **21**,811–819.

126 Kraus, T. M., Imhoff, F. B., Wexel, G., *et al.* Stem cells and basic fibroblast growth factor failed to improve tendon healing: an *in vivo* study using lentiviral gene transfer in a rat model. *The Journal of Bone and Joint Surgery. American Volume* (2014) **96**,761–769.

127 Gulotta, L. V., Kovacevic, D., Montgomery, S., *et al.* Stem cells genetically modified with the developmental gene MT1-MMP improve regeneration of the supraspinatus tendon-to-bone insertion site. *The American Journal of Sports Medicine* (2010) **38**,1429–1437.

128 Guo, C. A., Liu, X. G., Huo, J. Z., *et al.* Novel gene-modified-tissue engineering of cartilage using stable transforming growth factor-β1-transfected mesenchymal stem cells grown on chitosan scaffolds. *Journal of Bioscience and Bioengineering* (2007)**103**,547–556.

129 Pagnotto, M. R., Wang, Z., Karpie, J. C., *et al.* Adeno-associated viral gene transfer of transforming growth factor-beta1 to human mesenchymal stem cells improves cartilage repair. *Gene Therapy* (2007)**14**,804–813.

130 Xia, W., Jin, Y. Q., Kretlow, J. D., *et al.* Adenoviral transduction of hTGF-beta1 enhances the chondrogenesis of bone marrow derived stromal cells. *Biotechnology Letters* (2009)**31**,639–646.

131 Wu, W., Dan, Y., Yang, S. H., *et al.* Promotion of chondrogenesis of marrow stromal stem cells by TGF-β3 fusion protein *in vivo*. *Journal of Huazhong University of Science and Technology. Medical Sciences* (2013)**33**,692–699 (erratum in *Journal of Huazhong University of Science and Technology. Medical Sciences* (2014)**34**, 464).

132 Li, F., Jia, H., and Yu, C. ACL reconstruction in a rabbit model using irradiated Achilles allograft seeded with mesenchymal stem cells or PDGF-B gene-transfected mesenchymal stem cells. *Knee Surgery, Sports Traumatology, Arthroscopy* (2007)**15**,1219–1227.

133 Zhao, Z., Wang Z., Ge C. *et al.* Healing cranial defects with AdRunx2-transduced marrow stromal cells. *J Dent Res* (2007) **86**,1207–1211.

134 Gronthos, S., Chen, S., Wang, C. Y., *et al.* Telomerase accelerates osteogenesis of bone marrow stromal stem cells by upregulation of CBFA1, osterix, and osteocalcin. *Journal of Bone and Mineral Research* (2003)**18**,716–722.

135 Shi, S., Gronthos, S., Chen, S., *et al.* Bone formation by human postnatal bone marrow stromal stem cells is enhanced by telomerase expression. *Nature Biotechnology* (2002)**20**,587–591.

136 Tsuchiya, H., Kitoh, H., Sugiura, F., and Ishiguro, N. Chondrogenesis enhanced by overexpression of sox9 gene in mouse bone marrow-derived mesenchymal stem cells. *Biochemical and Biophysical Research Communications* (2003)**301**,338–343.

137 Hoffmann, A., Czichos, S., Kaps, C., *et al.* The T-box transcription factor Brachyury mediates cartilage development in mesenchymal stem cell line C3H10T1/2. *Journal of Cell Science* (2002) **115**,769–781.

138 Urs, S., Venkatesh, D., Tang, Y., *et al.* Sprouty1 is a critical regulatory switch of mesenchymal stem cell lineage allocation. *FASEB Journal* (2010)**24**,3264–3273.

139 Gulotta, L. V., Kovacevic, D., Packer, J. D., *et al.* Bone marrow-derived mesenchymal stem cells transduced with scleraxis improve rotator cuff healing in a rat model. *The American Journal of Sports Medicine* (2011)**39**,1282–1289.

140 Miura, K. and Oznono, K. Clinical condition and therapy of bone diseases. *Clinical Calcium* (2013)**23**,1789–1794 (in Japanese).

141 Cahill, R. A., Jones, O. Y., Klemperer, M., *et al.* Replacement of recipient stromal/mesenchymal cells after bone marrow transplantation using bone fragments and cultured osteoblast-like cells. *Biology of Blood and Marrow Transplantation* (2004)**10**,709–717.

142 Cahill, R. A., Wenkert, D., Perlman, S. A., *et al.* Infantile hypophosphatasia: transplantation therapy trial using bone fragments and cultured osteoblasts. *The Journal of Clinical Endocrinology and Metabolism* (2007)**92**,2923–2930.

143 Tadokoro, M., Machida, H., and Ohgushi, H. Genetic basis for skeletal disease. Stem cell therapy for genetic bone disorders. *Clinical Calcium* (2010)**20**,1228–1235 (in Japanese).

144 Horwitz, E. M., Gordon, P. L., Koo, W. K., *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proceedings of the National Academy of Sciences of the United States of America* (2002)**99**,8932–8937.

145 Chamberlain, J. R., Schwarze, U., Wang, P. R., *et al.* Gene targeting in stem cells from individuals with osteogenesis imperfecta. *Science* (2004)**303**,1198–1201.

146 Yang, S., Wei, D., Wang, D., *et al. In vitro* and *in vivo* synergistic interactions between the Runx2/Cbfa1 transcription factor and bone morphogenetic protein-2 in stimulating osteoblast differentiation. *Journal of Bone and Mineral Research* (2003)**18**,705–715.

147 Kumar, S., Wan, C., Ramaswamy, G., *et al.* Mesenchymal stem cells expressing osteogenic and angiogenic factors synergistically enhance bone formation in a mouse model of segmental bone defect. *Molecular Therapy* (2010)**18**,1026–1034.

148 Longobardi, L., O'Rear, L., Aakula, S., *et al.* Effect of IGF-I in the chondrogenesis of bone marrow mesenchymal stem cells in the presence or absence of TGF-β signaling. *Journal of Bone and Mineral Research* (2006)**21**,626–636.

149 Steinert, A. F., Palmer, G. D., Pilapil, C., *et al.* Enhanced *in vitro* chondrogenesis of primary mesenchymal stem cells by combined gene transfer. *Tissue Engineering. Part A* (2009)**15**,1127–1139.

150 Song, H., Song, B. W., Cha, M. J., *et al.* Modification of mesenchymal stem cells for cardiac regeneration. *Expert Opinion on Biological Therapy* (2010)**10**,309–319.

151 Nakanishi, C., Yamagishi, M., Yamahara, K., *et al.* Activation of cardiac progenitor cells through paracrine effects of mesenchymal stem cells. *Biochemical and Biophysical Research Communications* (2008)**374**,11–16.

152 Yang, F., Cho, S. W., Son, S. M., *et al.* Genetic engineering of human stem cells for enhanced angiogenesis using biodegradable polymeric nanoparticles. *Proceedings of the National Academy of Sciences of the United States of America* (2010)**107**,3317–3322.

153 Piao, W., Wang, H., Inoue, M., *et al.* Transplantation of Sendai viral angiopoietin-1-modified mesenchymal stem cells for ischemic limb disease. *Angiogenesis* (2010)**13**,203–210.

154 Manning, E., Pham, S., Li, S., *et al.* Interleukin-10 delivery via mesenchymal stem cells: a novel gene therapy approach to prevent lung ischemia–reperfusion injury. *Human Gene Therapy* (2010) **21**,713–727.

155 Winearls, C. G., Oliver, D. O., Pippard, M. J., *et al.* Effect of human erythropoietin derived from recombinant DNA on the anaemia of patients maintained by chronic haemodialysis. *Lancet* (1986) **2**,1175–1178.

156 Eliopoulos, N., Gagnon, R. F., Francois, M., and Galipeau, J. Erythropoietin delivery by genetically engineered bone marrow stromal cells for correction of anemia in mice with chronic renal failure. *Journal of the American Society of Nephrology* (2006)**17**,1576–1584.

157 Kucic, T., Copland, I. B., Cuerquis, J., *et al.* Mesenchymal stromal cells genetically engineered to overexpress IGF-I enhance cell-based gene therapy of renal failure-induced anemia. *American Journal of Physiology. Renal Physiology* (2008)**295**,F488–496.

158 Tögel, F., Hu, Z., Weiss, K., *et al.* Administered mesenchymal stem cells protect against ischemic acute renal failure through differentiation-independent mechanisms. *American Journal of Physiology. Renal Physiology* (2005)**289**,F31–42.

159 Hagiwara, M., Shen, B., Chao, L., and Chao, J. Kallikrein-modified mesenchymal stem cell implantation provides enhanced protection against acute ischemic kidney injury by inhibiting apoptosis and inflammation. *Human Gene Therapy* (2008)**19**,807–819.

160 Yuan, L., Wu, M. J., Sun, H. Y., *et al.* VEGF-modified human embryonic mesenchymal stem cell implantation enhances protection against cisplatin-induced acute kidney injury. *American Journal of Physiology. Renal Physiology* (2011)**300**,F207–F218.

161 Tanna, T. and Sachan, V. Mesenchymal stem cells: potential in treatment of neurodegenerative diseases. *Current Stem Cell Research & Therapy* (2014)**9**,513–521.

162 Crigler, L., Robey, R. C., Asawachaicharn, A., *et al.* Human mesenchymal stem cell subpopulations express a variety of neuro-regulatory molecules and promote neuronal cell survival and neuritogenesis. *Experimental Neurology* (2006)**198**,54–64.

163 Aizman, I., Tate, C. C., McGrogan, M., and Case C. C. Extracellular matrix produced by bone marrow stromal cells and by their derivative, SB623 cells, supports neural cell growth. *Journal of Neuroscience Research* (2009)**87**,3198–3206.

164 Zuccato, C., Ciammola, A., Rigamonti, D., *et al.* Loss of huntingtin-mediated *BDNF* gene transcription in Huntington's disease. *Science* (2001)**293**,493–498.

165 Boudreau, R. L., McBride, J. L., Martins, I., *et al.* Nonallele-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in Huntington's disease mice. *Molecular Therapy* (2009)**17**,1053–1063.

166 Olson, S. D., Kambal, A., Pollock, K., *et al.* Examination of mesenchymal stem cell-mediated RNAi transfer to Huntington's disease affected neuronal cells for reduction of huntingtin. *Molecular and Cellular Neurosciences* (2012)**49**,271–281.

167 Canals, J. M., Pineda, J. R., Torres-Peraza, J. F., *et al.* Brain-derived neurotrophic factor regulates the onset and severity of motor dysfunction associated with enkephalinergic neuronal degeneration in Huntington's disease. *The Journal of Neuroscience* (2004) **24**,7727–7739.

168 Dey, N. D., Bombard, M. C., Roland, B. P., *et al.* Genetically engineered mesenchymal stem cells reduce behavioral deficits in the YAC 128 mouse model of Huntington's disease. *Behavioural Brain Research* (2010)**214**,193–200.

169 Baydyuk, M. and Xu, B. BDNF signaling and survival of striatal neurons. *Frontiers in Cellular Neuroscience* (2014)**8**,254.

170 Zuccato, C., Valenza, M., and Cattaneo, E. Molecular mechanisms and potential therapeutical targets in Huntington's disease. *Physiological Reviews* (2010)**90**,905–981.

171 Vucic, S. and Kiernan, M. C. Pathophysiology of neurodegeneration in familial amyotrophic lateral sclerosis. *Current Molecular Medicine* (2009)**9**,255–272.

172 Acsadi, G., Anguelov, R. A., Yang, H., *et al.* Increased survival and function of SOD1 mice after glial cell-derived neurotrophic factor gene therapy. *Human Gene Therapy* (2002)**13**,1047–1059.

173 Suzuki, M., McHugh, J., Tork, C., *et al.* Direct muscle delivery of GDNF with human mesenchymal stem cells improves motor neuron survival and function in a rat model of familial ALS. *Molecular Therapy* (2008)**16**,2002–2010.

174 Funakoshi, H., Ohya, W., Kadoyama, K., and Nakamura, T. ALS and neurotrophic factors – HGF as a novel neurotrophic and neuroregenerative factor. *Brain and Nerve* (2007)**59**,1195–1202.

175 Nagano, I., Ilieva, H., Shiote, M., *et al.* Therapeutic benefit of intrathecal injection of insulin-like growth factor-1 in a mouse model of amyotrophic lateral sclerosis. *Journal of the Neurological Sciences* (2005)**235**,61–68.

176 Kim, H. Y., Kim, H., Oh, K. W., *et al.* Biological markers of mesenchymal stromal cells as predictors of response to autologous stem cell transplantation in patients with amyotrophic lateral sclerosis: an investigator-initiated trial and *in vivo* study. *Stem Cells* (2014)**32**,2724–2731.

177 Schapira, A. H. and Jenner, P. Etiology and pathogenesis of Parkinson's disease. *Movement Disorders* (2011)**26**,1049–1055.

178 Peterson, A. L. and Nutt, J. G. Treatment of Parkinson's disease with trophic factors. *Neurotherapeutics* (2008)**5**,270–280.

179 Glavaski-Joksimovic, A., Virag, T., Mangatu, T. A., *et al.* Glial cell line-derived neurotrophic factor-secreting genetically modified human bone marrow-derived mesenchymal stem cells promote recovery in a rat model of Parkinson's disease. *Journal of Neuroscience Research* (2010)**88**,2669–2681.

180 Somoza, R., Juri, C., Baes, M., *et al.* Intranigral transplantation of epigenetically induced BDNF-secreting human mesenchymal stem cells: implications for cell-based therapies in Parkinson's disease. *Biology of Blood and Marrow Transplantation* (2010) **16**,1530–1540.

181 Li, J. Y., Englund, E., Holton, J. L., *et al.* Lewy bodies in grafted neurons in subjects with Parkinson's disease suggest host-to-graft disease propagation. *Nature Medicine* (2008)**14**,501–503.

182 Kashiwakura, Y., Ohmori, T., Mimuro, J., *et al.* Intra-articular injection of mesenchymal stem cells expressing coagulation factor ameliorates hemophilic arthropathy in factor VIII-deficient mice. *Journal of Thrombosis and Haemostasis* (2012)**10**,1802–1813.

183 Dodd, M., Marquez-Curtis, L., Janowska-Wieczorek, A., and Hortelano, G. Sustained expression of coagulation factor IX by modified cord blood-derived mesenchymal stromal cells. *The Journal of Gene Medicine* (2014)**16**,131–142.

184 Koç, O. N., Day, J., Nieder, M., *et al.* Allogeneic mesenchymal stem cell infusion for treatment of metachromatic leukodystrophy

(MLD) and Hurler syndrome (MPS-IH). *Bone Marrow Transplantation* (2002)**30**,215–222.

185 Boroujeni, Z. N. and Aleyasin, A. Insulin producing cells established using non-integrated lentiviral vector harboring PDX1 gene. *World Journal of Stem Cells* (2013)**5**,217–228.

186 Li, Y., Zhang, R., Qiao, H., *et al.* Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. *Journal of Cellular Physiology* (2007)**211**,36–44.

187 Gao, J., Dennis, J. E., Muzic, R. F., *et al.* The dynamic *in vivo* distribution of bone marrow-derived mesenchymal stem cells after infusion. *Cells, Tissues, Organs* (2001)**169**,12–20.

188 Fink Jr, D. W. FDA regulation of stem cell-based products. *Science* (2009)**324**,1662–1663.

189 Haque, N., Rahman, M. T., Abu Kasim, N. H., *et al.* Hypoxic culture conditions as a solution for mesenchymal stem cell based regenerative therapy. *The Scientific World Journal* (2013)**2013**,632972.

190 Beegle, J., Lakatos, K., Kalomoiris, S., *et al.* Hypoxic preconditioning of mesenchymal stromal cells induces metabolic changes that enhance survival and promote cell retention *in vivo*. *Stem Cells* (2015)**33**,1818–1828.

191 Di Stasi, A., Tey, S. K., Dotti, G., *et al.* Inducible apoptosis as a safety switch for adoptive cell therapy. *The New England Journal of Medicine* (2011)**365**,1673–1683.

192 Straathof, K. C., Pulè, M. A., Yotnda, P., *et al.* An inducible caspase 9 safety switch for T-cell therapy. *Blood* (2005)**105**,4247–4254.

193 Choi, Y. H., Kurtz, A., and Stamm, C. Mesenchymal stem cells for cardiac cell therapy. *Human Gene Therapy* (2011)**22**,3–17.

194 Miettinen, J. A., Ylitalo, K. V., Niemelä, M., *et al.* Left ventricular functional recovery after intracoronary injection of autologous bone marrow-derived stem cells in patients with acute myocardial infarction: a dose–response pilot study. *International Journal of Cardiology* (2012)**154**,354–356.

195 Hare, J. M., Traverse, J. H., Henry, T. D., *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (prochymal) after acute myocardial infarction. *Journal of the American College of Cardiology* (2009)**54**,2277–2286.

196 Kebriaei, P., Isola, L., Bahceci, E., *et al.* Adult human mesenchymal stem cells added to corticosteroid therapy for the treatment of acute graft-versus-host disease. *Biology of Blood and Marrow Transplantation* (2009)**15**,804–811.

197 Connick, P., Kolappan, M., Crawley, C., *et al.* Autologous mesenchymal stem cells for the treatment of secondary progressive multiple sclerosis: an open-label phase 2a proof-of-concept study. *The Lancet. Neurology* (2012)**11**,150–156.

198 Richardson, J. D., Bertaso, A. G., Psaltis, P. J., *et al.* Impact of timing and dose of mesenchymal stromal cell therapy in a preclinical model of acute myocardial infarction. *Journal of Cardiac Failure* (2013)**19**,342–353.

199 Sayyar, B., Dodd, M., Marquez-Curtis, L., *et al.* Cell-matrix interactions of factor IX (FIX)-engineered human mesenchymal stromal cells encapsulated in RGD-alginate vs. fibrinogen-alginate microcapsules. *Artificial Cells, Nanomedicine, and Biotechnology* (2014) **42**,102–109.

CHAPTER 62

# Gene therapy for cancer using mesenchymal stromal cells

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## 62.1 Introduction

Mesenchymal stromal cells (MSCs) are a promising platform for cellular and genetic therapy and regenerative medicine. MSCs are nonhematopoietic progenitor cells that can be easily isolated from a variety of tissues, including bone marrow [1], adipose tissue [1–3], the placenta and umbilical cord [1,4,5], Wharton's jelly [6], tendons [7], and dental pulp [8]. Furthermore, MSCs can be easily expanded, genetically modified and have the ability to differentiate *in vitro* into mesenchymal lineages, including adipocytes, osteocytes, chondrocytes, muscle cells and stromal cells [9].

### 62.1.1 Biological characteristics of mesenchymal stromal cells

The biological characteristics of MSCs have generated markedly growing interest in the field of cellular therapy (Figure 62.1).

However, investigators have reported studies of MSCs using different methods for cell isolation and expansion, and different approaches for characterizing these cells. Thus, it is difficult to compare and contrast study outcomes, which has hindered progress in the field. To better address this issue the International Society for Cellular Therapy proposed minimal criteria to identify human MSCs as follows: (1) MSCs must be plastic-adherent when maintained in standard culture conditions; (2) MSCs must express CD73, CD90 and CD105, and lack expression of CD45, CD34, CD14, CD11b, CD79, CD19, or human leukocyte antigen (HLA)-DR on the cell surface; and (3) MSCs must differentiate *in vitro* into cells of the mesenchymal lineage, including osteoblasts, adipocytes, and chondroblasts [10,11]. This area took another significant step forward with the publication in 2013 of a joint statement from the International Federation for Adipose Therapeutics and Science and the International Society for Cellular Therapy on stromal cells from adipose-tissue-derived stromal vascular fraction and culture-expanded adipose-tissue-derived stromal/stem cells [12]. The centrifuged but uncultured stromal vascular fraction was identified as being $CD45^-$, $CD235a^-$ (glycophorin A), $CD31^-$ (platelet endothelial cell adhesion molecule) and $CD34^+$, while cell surface positivity for CD13 (alanine aminopeptidase), CD73, CD90, and CD105 were best used in conjunction with CD45, CD235a, and CD31 [12]. *Ex-vivo*-expanded adipose-tissue-derived stromal/stem cells retain cell surface markers expressed by other mesenchymal stromal/stem cells, including CD44 (the hyaluronic acid receptor), CD73, CD90, and CD105 and are also negative for CD45 and CD31, but can be distinguished from bone-marrow-derived MSCs by being $CD36^+$ (fatty acid translocase, also

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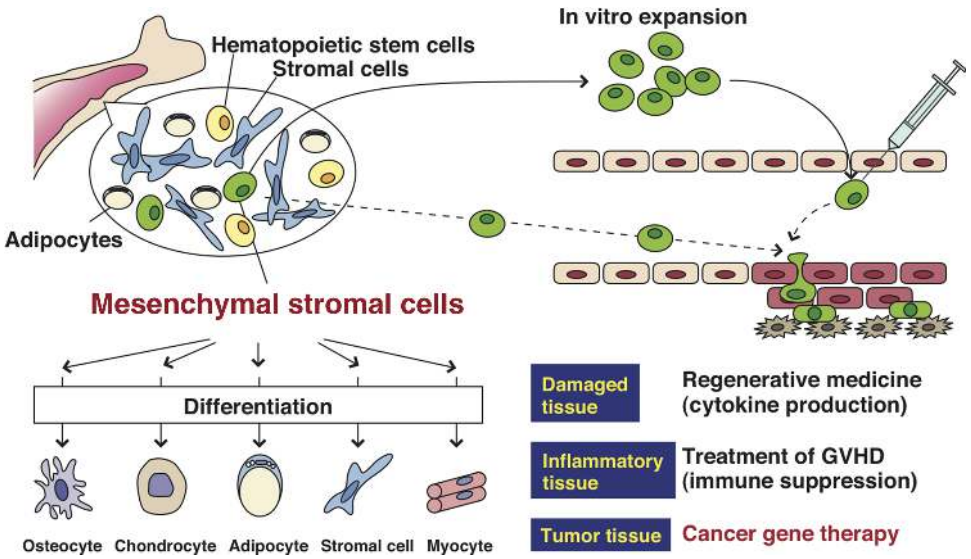



**Figure 62.1** Concepts in the use of MSCs for regenerative medicine and cell therapy. In the bone marrow, there are different types of tissue stem cells, including hematopoietic stem cells and MSCs. MSCs account for a small population of total bone marrow cells, and they can differentiate into various different mesenchymal lineages. MSCs can also accumulate at sites of (i) tissue damage, (ii) tissue inflammation, and (iii) tumors. Therefore, MSCs can be used for (i) regenerative therapy, (ii) treatment of graft-versus-host disease (GVHD), and (iii) cancer gene and cell therapy (targeted delivery). (See color plate section.)

known as glycoprotein IIIb) and $CD106^{-}$ (vascular cell-adhesion molecule 1 (VCAM-1)) [12].

### 62.1.2 Immunomodulatory effects of mesenchymal stromal cells on immune cells

Another feature of MSCs is their low immunogenicity probably because of the absence of costimulatory molecules on their cell surface [13]. Thus, MSCs do not activate the host adaptive immune response and can escape immunological rejection when transferred into unrelated HLA-nonidentical recipients (see Chapter 57). These characteristics permit the infusion of MSCs into recipients without HLA matching. Interestingly, MSCs exhibit a homing predisposition toward damaged and inflamed tissues. MSCs may be useful in the prevention or treatment of GVHD, a significant cause of morbidity and mortality following allogeneic hematopoietic stem cell transplantation, although phase III clinical trials in this area are ongoing. Human MSCs suppress T cell proliferation induced by allogeneic peripheral blood lymphocytes or phytohemagglutinin [14]. Le Blanc *et al.* reported the index case of a patient with severe acute GVHD following bone marrow transplantation, which was refractory to all conventional immune suppressive agents, but which showed marked improvement following two intravenous infusions of haploidentical (maternal) MSCs [15].

### 62.1.3 Tumor homing of mesenchymal stromal cells

Tumors have been referred to as "wounds that never heal" [16]. Inflammatory immune cells such as neutrophils, macrophages, and natural killer cells are recruited to the tumor site. Tumor growth can be promoted by inflammation. MSCs also exhibit tropism for tumor tissues and can be utilized as a cellular vehicle for anticancer gene delivery to tumor sites [17]. This type of targeted therapy can reduce the side effects of anticancer agents by facilitating an effective concentration of these agents at local tumor sites.

## 62.2 Applications of genetically engineered mesenchymal stromal cells for cancer therapy

Since it was first reported that MSCs show tropism for tumors [17], many studies have demonstrated that genetically modified MSCs either overexpress therapeutic gene products or demonstrate tumor tropism. In this section we summarize the various therapeutic gene products that have been successfully delivered to tumors using MSCs as cellular delivery vehicles (Table 62.1).

### 62.2.1 Interferons

An early application of MSCs for targeted cancer therapy was the directed delivery of IFN-β [17–20]. MSCs were transduced with the gene encoding IFN-β, followed by their infusion into mice harboring tumor xenografts. MSC treatment resulted in reduced tumor growth and the prolonged survival of tumor-bearing mice. Based on this observation, genetically modified MSCs from various tissues have been evaluated for their therapeutic efficacy and ability to act as cellular vehicles.

**Table 62.1** Therapeutic gene products successfully delivered to tumors using MSCs as a cellular delivery vehicle.

| Agent | Rationale | Model | Reference |
|---|---|---|---|
| IFN-β | Induces differentiation, S-phase accumulation, and apoptosis | Metastasis (prostate, breast, melanoma) | [17–19] |
| | | Orthotopic (glioma) | [20] |
| IL-2 | Immunomodulation | Orthotopic (glioma) | [21] |
| IL-12 | Activates CTLs and NK cells and produces IFN-γ | Subcutaneous (melanoma, hepatoma, lung) | [22,23] |
| CX3CL1 | Activates CTLs and NK cells | Metastasis (melanoma, colon) | [24] |
| GCV/HSV-TK | Prodrug conversion | Subcutaneous, orthotopic (glioma) | [25,26] |
| 5-FC/CD | Prodrug conversion (5-FC →5-FU) | Subcutaneous (melanoma, colon) | [27–30] |
| NK4 | Inhibits angiogenesis and promotes apoptosis | Metastasis (colon) | [31] |
| TRAIL | Induces apoptosis | Subcutaneous (breast)<br>Metastasis (breast)<br>Orthotopic (glioma) | [32,33] |
| Oncolytic viruses | Destroys tumors by viral replication | Orthotopic (breast, lung, ovarian)<br>Metastasis (breast) | [34–36] |

5-FC: 5-fluorocytosine; 5-FU: 5-fluorouracil; CD: cytosine deaminase; CTL: cytotoxic T lymphocyte; CX3CL1: chemokine C-X3-C motif ligand 1; GCV: ganciclovir; HSV-TK: herpes simplex virus–thymidine kinase; IFN: interferon; IL: interleukin; NK: natural killer; TRAIL: tumor-necrosis-factor-related apoptosis-inducing ligand.

### 62.2.2 Interleukins

ILs are molecules that regulate the inflammatory and immune responses, and are often used as therapeutic agents. IL-2-expressing MSCs have been used to improve immunological surveillance of tumors by activating CTLs and NK cells [21]. Administration of IL-12-expressing MSCs has been shown to suppress metastasis and increase tumor cell apoptosis in mice bearing melanoma [22], lung cancer, or hepatoma [23].

### 62.2.3 Chemokines

The antitumor effect of CX3CL1 (also known as fractalkine), which is a chemoattractant for T cells and monocytes, has also been tested. MSCs expressing CX3CL1 induced both innate and adoptive immunity, inhibited lung metastases of melanoma, and prolonged survival of tumor-bearing mice [24].

### 62.2.4 Suicide genes

Suicide gene cancer therapy has been considered for the treatment of invasive tumors such as malignant glioma in the brain [25,26]. Many prodrug activation techniques that convert a nontoxic prodrug into a toxic antimetabolite are available for the selective killing of tumor cells. HSV-TK and CD, which confer sensitivity to GCV and 5-FU respectively, are being evaluated in clinical trials. When HSV-TK-expressing MSCs [27,28] or CD-expressing MSCs [29,30] were injected into tumor-bearing mice, the tumor volume was reduced through a bystander-mediated tumor cell killing following the administration of GCV or 5-FC. We have developed genetically modified MSCs that produce retroviral vectors that encode HSV-TK with the aim of augmenting the therapeutic efficacy of suicide cancer gene therapy [27]. MSCs isolated from the bone marrow of Sprague–Dawley rats were nucleofected with proviral plasmid DNA that expressed either HSV-TK alone (non-VP-MSCs), vesicular stomatitis virus-glycoprotein (VSV-G), protein-pseudotyped long-terminal-repeat-HSV-TK with Gag-pol and VSVG, or whole retroviral vector components. (Nucleofection is an electroporation-based transfection method that enables transfer of nucleic acids such as DNA and RNA into cells by applying a specific voltage and reagents.) Tumor growth was more efficiently suppressed after the injection of VP-MSCs compared with non-VP-MSCs. This efficacy was dependent on therapeutic gene amplification through the production of retrovirus progeny from MSCs and the transduction of tumor cells *in situ*.

### 62.2.5 Other approaches

The efficacy of other therapeutic genes, including NK4 [31] and TRAIL [32,33], has also been examined in various tumor models. NK4, a competitive antagonist of hepatocyte growth factor (HGF) and its receptor c-Met tyrosine kinase, is an internal fragment of HGF and is composed of the N-terminal and four kringle domains. Oncolytic virotherapy is emerging as a promising strategy for tumor treatment. MSCs can be used as carriers to deliver oncolytic viruses to tumor sites [34–36]. Experimentally, the injection of genetically modified MSCs can result in tumor growth inhibition, metastasis suppression, and prolonged survival.

## 62.3 Molecular mechanisms of mesenchymal stromal cell accumulation at tumor sites

As already described, the use of MSCs to deliver anticancer agents is an attractive novel cancer therapeutic strategy. This type of targeted therapy can reduce the systemic side effects of

anticancer agents by increasing their effective concentration at local tumor sites without elevating systemic concentrations. However, how and why MSCs accumulate at tumor sites remains poorly understood. Endothelial cells (ECs), pericytes, and stromal cells are all known to support tumor growth and contribute to the tumor microenvironment by producing various growth factors, including vascular endothelial growth factor-A, IL-8, transforming growth factor-β, epidermal growth factor, and platelet-derived growth factor [37–42]. MSCs can also act in a similarly supportive fashion. It can be speculated that MSCs, when they accumulate at tumor sites, differentiate into pericytes or cancer-associated fibroblasts, and can be involved in facilitating tumor growth by providing structural support to the tumor microenvironment [17,43,44]. Interestingly, breast cancer cells stimulate MSCs, and MSCs in turn support cancer cell invasion and metastasis by secreting the chemokine CCL5 (also known as RANTES) [45]. Conversely, MSCs can also induce apoptosis in tumor cells by blocking the phosphorylation of AKT [46] or preventing cell cycle progression [47]. Moreover, MSCs can produce Dickkopf 1 to attenuate the potential for tumor cell growth and malignancy [48]. Because MSCs have dual and opposing effects on tumor growth, modification with antitumor genes is required for therapeutic MSC-based cancer targeting. Furthermore, it is also necessary to promote more efficient MSC accumulation at tumor sites.

### 62.3.1 Migratory factors

If it becomes possible to increase the efficiency of MSC accumulation at tumor sites, MSCs could effectively target not only primary tumors but also metastatic lesions. It is thought that MSCs home to damaged tissues, such as injury or inflammation, by the release of inflammatory cytokines. Tumors contain a microenvironment consisting of many inflammatory cells [49]. This microenvironment promotes the recruitment of MSCs because of the various soluble factors that are secreted by both tumor and inflammatory cells, including tumor necrosis factor (TNF)-α, IL-6 [50,51], IL-8 [52,53], transforming growth factor-β [54], placental growth factor [55], platelet-derived growth factor [56], HGF [56], stromal-cell-derived factor-1 [56–58], matrix metalloproteinase (MMP) [59], chemokines [51,54], monocyte chemoattractant protein-1 (MCP-1) [53,60], and proinflammatory peptide LL-37 [61]. However, we found that although systemically injected MSCs accumulated at tumor sites, subcutaneously injected MSCs did not. We also compared the migration capacity of MSCs and fibroblasts toward growth factors and chemokines *in vitro* and found that the chemoattraction of fibroblasts toward these factors was stronger than that of MSCs [56]. These findings suggest that the mechanism of MSC accumulation cannot be solely explained by cytokine-mediated migration.

### 62.3.2 Interactions between mesenchymal stromal cells and endothelial cells

The tumors generated in the studies just described had tumor stroma with many blood vessels, and MSCs in particular accumulated at the boundaries between tumors and tumor stroma [56]. Furthermore, MSC accumulation at the tumor sites was only observed when cells were injected into the left ventricle of the heart. We therefore focused on MSC–EC adhesion to elucidate the mechanisms involved. Interestingly, the stimulation of MSCs by TNF-α enhanced the adhesion of MSCs to ECs *in vitro*. This adhesion could be partially inhibited by antibodies that block VCAM-1 and very late antigen-4 (VLA-4). It is well known that TNF-α can induce VCAM-1 expression via the NF-κB (nuclear factor kappa light chain-enhancer of activated B cells) signaling pathway [62,63]. Parthenolide has anti-inflammatory activities and can suppress NF-κB activity by inhibiting IκBα (inhibitor of kappa Bα) phosphorylation following stimulation by TNF-α [64,65], and parthenolide strongly inhibited TNF-α-induced VCAM-1 expression on MSCs [56]. *In vivo* imaging using luciferase-expressing MSCs showed that bioluminescent signals gradually increased at tumor sites in mice injected with untreated MSCs. However, we observed very weak signals at tumor sites in mice injected with parthenolide-treated MSCs. These findings suggest that NF-κB activity can regulate MSC accumulation at tumor sites by inducing VCAM-1 expression and subsequently promoting cellular interactions with tumor vessel ECs (Figure 62.2).

Although we focused on the role of TNF-α, other inflammatory cytokines, including IL-1β [66,67] and IFN-γ [68], can also induce VCAM-1 expression on target cells and might also be involved in MSC accumulation.

## 62.4 Considerations in the use of genetically engineered mesenchymal stromal cells in cancer therapy

As described, activation with TNF-α might be a critically important factor in MSC accumulation. TNF-α is a major inflammatory cytokine that has important roles in diverse cellular events, including cell survival, proliferation, differentiation, and death [69–72]. Numerous reports have documented elevated TNF-α levels in the serum or plasma of people with cancer, and TNF-α levels correlate closely with tumor progression and metastasis [69,73–75]. For example, TNF-α readily induces IL-6 and MCP-1 secretion by cancer-associated fibroblasts and normal fibroblasts, and it also has an indirect role in generating a prometastatic microenvironment. Furthermore, TNF-α is also released in tissues undergoing inflammation, including myocardial infarction [76,77] and GVHD [78]. MSCs accumulate in these inflamed tissues. Thus, proinflammatory cytokines also promote the homing of MSCs to these tissues, and MSC-based tissue-targeting strategies could be adapted for various inflammation-related disorders. Furthermore, MSCs can induce neovascularization and immunosuppression, thereby protecting tissues from disease-related damage.

For MSC-based cancer-targeted gene therapies it is thought that the therapeutic efficacy is directly related to the degree of

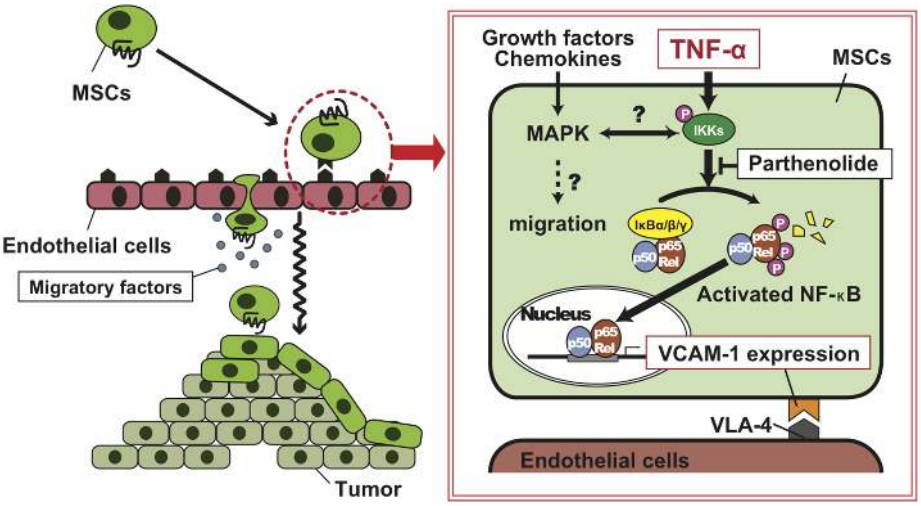



**Figure 62.2** A proposed mechanism for MSC accumulation at tumor sites. Growth factors and chemokines recruit MSCs to the tumor microenvironment. Inflammatory cytokines, such as TNF-α, stimulate MSCs and induce cell surface VCAM-1 expression. Next, activated MSCs attach to the tumor vasculature. Subsequently, migratory factors, such as chemokines or cytokines, enhance MSC migration. Finally, MSCs accumulate at tumor sites. IKKs: IκB kinases; MAPK: mitogen-activated protein kinase. (See color plate section.)

MSC accumulation at tumor sites. Data from our laboratory suggest that the combined use of NF-κB inhibitors, such as bortezomib [79], or TNF-α blocking agents [80], such as infliximab, etanercept, adalimumab, golimumab, and certolizumab, can reduce the therapeutic efficacy of genetically modified MSCs because of the inhibition of MSC accumulation at tumor sites. Interestingly, other data suggest that targeted approaches to neutralize TNF in patients with heart failure can result in exacerbated disease [81]. In contrast, tumor-specific TNF-α-inducing agents would be useful for enhancing therapeutic efficacy. Thus, further investigation is warranted for the identification of such agents to establish safer and more effective therapeutic strategies.

## 62.5 Summary and conclusions

Cellular and gene therapies represent promising new treatment strategies in medicine. Hematopoietic stem cells, lymphocytes, and MSCs can all be used as cell sources for use in such therapies. Strategies for gene therapy are often based on those of cell therapy, and some examples are expected to be clinically implemented in the near future. MSCs can be used to improve stromal cell engraftment and the acceleration of hematopoietic reconstitution based on their hematopoiesis-supporting ability (see Chapter 32). Furthermore, MSCs may be useful in the treatment of severe GVHD because of their immunosuppressive ability. This application is also based on their ability to home to damaged and inflammatory tissues. Interestingly, MSCs also show tumor-homing ability, allowing them to be used to directly deliver anticancer genes to tumors. MSC accumulation at tumor sites is related to the migratory capacity of MSCs toward growth factors and chemokines, but also to MSC–EC adhesion following stimulation by TNF-α (Figure 61.2). Furthermore, NF-κB activity can regulate MSC accumulation at tumor sites through the induction of VCAM-1 expression, which results in interactions with tumor blood vessel endothelial cells. Although MSCs are useful as cellular vehicles for cancer-targeted gene therapy, strategies to increase MSC accumulation are required to enhance their therapeutic efficacy. Thus, strategies to enhance local accumulation of MSCs should also be developed.

## References

1 Kern S, Eichler H, Stoeve J, *et al.* Comparative analysis of mesenchymal stem cells from bone marrow, umbilical cord blood, or adipose tissue. *Stem Cells* 2006;**24**:1294–1301.
2 Zuk PA, Zhu M, Ashjian P, *et al.* Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell* 2002;**13**:4279–4295.
3 Zannettino AC, Paton S, Arthur A, *et al.* Multipotential human adipose-derived stromal stem cells exhibit a perivascular phenotype *in vitro* and *in vivo*. *J Cell Physiol* 2008;**214**:413–421.
4 Igura K, Zhang X, Takahashi K, *et al.* Isolation and characterization of mesenchymal progenitor cells from chorionic villi of human placenta. *Cytotherapy* 2004;**6**:543–553.
5 Romanov YA, Svintsitskaya VA, Smirnov VN. Searching for alternative sources of postnatal human mesenchymal stem cells: candidate MSC-like cells from umbilical cord. *Stem Cells* 2003;**21**:105–110.
6 Wang HS, Hung SC, Peng ST, *et al.* Mesenchymal stem cells in the Wharton's jelly of the human umbilical cord. *Stem Cells* 2004;**22**:1330–1337.
7 Bi Y, Ehirchiou D, Kilts TM, *et al.* Identification of tendon stem/progenitor cells and the role of the extracellular matrix in their niche. *Nat Med* 2007;**13**:1219–1227.

8 Gronthos S, Mankani M, Brahim J, *et al.* Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo. *Proc Natl Acad Sci U S A* 2000;**97**:13625–13630.

9 Pittenger MF, Mackay AM, Beck SC, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.

10 Dominici M, Le Blanc K, Muller I, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.

11 Horwitz EM, Le Blanc K, Dominici M, *et al.* Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement. *Cytotherapy* 2005;**7**:393–395.

12 Bourin P, Bunnell BA, Casteilla L, *et al.* Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics (IFATS) and Science and the International Society for Cellular Therapy (ISCT). *Cytotherapy* 2013;**15**:641–648.

13 Nauta AJ, Fibbe WE. Immunomodulatory properties of mesenchymal stromal cells. *Blood* 2007;**110**:3499–3506.

14 Di Nicola M, Carlo-Stella C, Magni M, *et al.* Human bone marrow stromal cells suppress T-lymphocyte proliferation induced by cellular or nonspecific mitogenic stimuli. *Blood* 2002;**99**:8932–8937.

15 Le Blanc K, Rasmusson I, Sundberg B, *et al.* Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004;**363**:1439–1441.

16 Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* 1986;**315**:1650–1659.

17 Studeny M, Marini FC, Champlin RE, *et al.* Bone marrow-derived mesenchymal stem cells as vehicles for interferon-β delivery into tumors. *Cancer Res* 2002;**62**:3603–3608.

18 Studeny M, Marini FC, Dembinski JL, *et al.* Mesenchymal stem cells: potential precursors for tumor stroma and targeted-delivery vehicles for anticancer agents. *J Natl Cancer Inst* 2004;**96**:1593–1603.

19 Ren C, Kumar S, Chanda D, *et al.* Cancer gene therapy using mesenchymal stem cells expressing interferon-β in a mouse prostate cancer lung metastasis model. *Gene Ther* 2008;**15**:1446–1453.

20 Nakamizo A, Marini F, Amano T, *et al.* Human bone marrow-derived mesenchymal stem cells in the treatment of gliomas. *Cancer Res* 2005;**65**:3307–3318.

21 Nakamura K, Ito Y, Kawano Y, *et al.* Antitumor effect of genetically engineered mesenchymal stem cells in a rat glioma model. *Gene Ther* 2004;**11**:1155–1164.

22 Chen X, Lin X, Zhao J, *et al.* A tumor-selective biotherapy with prolonged impact on established metastases based on cytokine gene-engineered MSCs. *Mol Ther* 2008;**16**:749–756.

23 Chen XC, Wang R, Zhao X, *et al.* Prophylaxis against carcinogenesis in three kinds of unestablished tumor models via IL12-gene-engineered MSCs. *Carcinogenesis* 2006;**27**:2434–2441.

24 Xin H, Kanehira M, Mizuguchi H, *et al.* Targeted delivery of CX3CL1 to multiple lung tumors by mesenchymal stem cells. *Stem Cells* 2007;**25**:1618–1626.

25 Ram Z, Culver KW, Oshiro EM, *et al.* Therapy of malignant brain tumors by intratumoral implantation of retroviral vector-producing cells. *Nat Med* 1997;**3**:1354–1361.

26 Rainov NG. A phase III clinical evaluation of herpes simplex virus type 1 thymidine kinase and ganciclovir gene therapy as an adjuvant to surgical resection and radiation in adults with previously untreated glioblastoma multiforme. *Hum Gene Ther* 2000;**11**:2389–2401.

27 Uchibori R, Okada T, Ito T, *et al.* Retroviral vector-producing mesenchymal stem cells for targeted suicide cancer gene therapy. *J Gene Med* 2009;**11**:373–381.

28 Matuskova M, Hlubinova K, Pastorakova A, *et al.* HSV-tk expressing mesenchymal stem cells exert bystander effect on human glioblastoma cells. *Cancer Lett* 2009;**290**:58–67.

29 Kucerova L, Matuskova M, Pastorakova A, *et al.* Cytosine deaminase expressing human mesenchymal stem cells mediated tumour regression in melanoma bearing mice. *J Gene Med* 2008;**10**:1071–1082.

30 Kucerova L, Altanerova V, Matuskova M, *et al.* Adipose tissue-derived human mesenchymal stem cells mediated prodrug cancer gene therapy. *Cancer Res* 2007;**67**:6304–6313.

31 Kanehira M, Xin H, Hoshino K, *et al.* Targeted delivery of NK4 to multiple lung tumors by bone marrow-derived mesenchymal stem cells. *Cancer Gene Ther* 2007;**14**:894–903.

32 Kim SM, Lim JY, Park SI, *et al.* Gene therapy using TRAIL-secreting human umbilical cord blood-derived mesenchymal stem cells against intracranial glioma. *Cancer Res* 2008;**68**:9614–9623.

33 Loebinger MR, Eddaoudi A, Davies D, Janes SM. Mesenchymal stem cell delivery of TRAIL can eliminate metastatic cancer. *Cancer Res* 2009;**69**:4134–4142.

34 Komarova S, Kawakami Y, Stoff-Khalili MA, *et al.* Mesenchymal progenitor cells as cellular vehicles for delivery of oncolytic adenoviruses. *Mol Cancer Ther* 2006;**5**:755–766.

35 Hakkarainen T, Särkioja M, Lehenkari P, *et al.* Human mesenchymal stem cells lack tumor tropism but enhance the antitumor activity of oncolytic adenoviruses in orthotopic lung and breast tumors. *Hum Gene Ther* 2007;**18**:627–641.

36 Stoff-Khalili MA, Rivera AA, Mathis JM, *et al.* Mesenchymal stem cells as a vehicle for targeted delivery of CRAds to lung metastases of breast carcinoma. *Breast Cancer Res Treat* 2007;**105**:157–167.

37 Benjamin LE, Golijanin D, Itin A, *et al.* Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 1999;**103**:159–165.

38 Song S, Ewald AJ, Stallcup W, *et al.* PDGFRβ$^{+}$ perivascular progenitor cells in tumours regulate pericyte differentiation and vascular survival. *Nat Cell Biol* 2005;**7**:870–879.

39 Bababeygy SR, Cheshier SH, Hou LC, *et al.* Hematopoietic stem cell-derived pericytic cells in brain tumor angio-architecture. *Stem Cells Dev* 2008;**17**:11–18.

40 Lyden D, Hattori K, Dias S, *et al.* Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 2001;**7**:1194–1201.

41 Peters BA, Diaz LA, Polyak K, *et al.* Contribution of bone marrow-derived endothelial cells to human tumor vasculature. *Nat Med* 2005;**11**:261–262.

42 Spring H, Schüler T, Arnold B, *et al.* Chemokines direct endothelial progenitors into tumor neovessels. *Proc Natl Acad Sci U S A* 2005;**102**:18111–18116.

43 Bexell D, Gunnarsson S, Tormin A, *et al.* Bone marrow multipotent mesenchymal stroma cells act as pericyte-like migratory vehicles in experimental gliomas. *Mol Ther* 2009;**17**:183–190.

44 Bexell D, Scheding S, Bengzon J. Toward brain tumor gene therapy using multipotent mesenchymal stromal cell vectors. *Mol Ther* 2010;**18**:1067–1075.

45 Karnoub AE, Dash AB, Vo AP, *et al*. Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. *Nature* 2007;**449**:557–563.

46 Khakoo AY, Pati S, Anderson SA, *et al*. Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. *Exp Med* 2006;**203**:1235–1247.

47 Lu YR, Yuan Y, Wang XJ, *et al*. The growth inhibitory effect of mesenchymal stem cells on tumor cells *in vitro* and *in vivo*. *Cancer Biol Ther* 2008;**7**:245–251.

48 Etheridge SL, Spencer GJ, Heath DJ, Genever PG. Expression profiling and functional analysis of Wnt signaling mechanisms in mesenchymal stem cells. *Stem Cells* 2004;**22**:849–860.

49 Coussens LM, Werb Z. Inflammation and cancer. *Nature* 2002;**420**:860–867.

50 Liu S, Ginestier C, Ou SJ, *et al*. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer Res* 2011;**15**:614–624.

51 Rattigan Y, Hsu JM, Mishra PJ, *et al*. Interleukin 6 mediated recruitment of mesenchymal stem cells to the hypoxic tumor milieu. *Exp Cell Res* 2010;**316**:3417–3424.

52 Senst C, Nazari-Shafti T, Kruger S, *et al*. Prospective dual role of mesenchymal stem cells in breast tumor microenvironment. *Breast Cancer Res Treat* 2013;**137**:69–79.

53 Wang L, Li Y, Chen X, *et al*. MCP-1, MIP-1, IL-8 and ischemic cerebral tissue enhance human bone marrow stromal cell migration in interface culture. *Hematology* 2002;**7**:113–117.

54 Goldstein RH, Reagan MR, Anderson K, *et al*. Human bone marrow-derived MSCs can home to orthotopic breast cancer tumors and promote bone metastasis. *Cancer Res* 2010;**70**:10044–10050.

55 Chaturvedi P, Gilkes DM, Wong CC, *et al*. Hypoxia-inducible factor-dependent breast cancer-mesenchymal stem cell bidirectional signaling promotes metastasis. *J Clin Invest* 2013;**123**:189–205.

56 Uchibori R, Tsukahara T, Mizuguchi H, *et al*. NF-κB activity regulates mesenchymal stem cell accumulation at tumor sites. *Cancer Res* 2013;**73**:364–372.

57 Lin G, Yang R, Banie L, *et al*. Effects of transplantation of adipose tissue-derived stem cells on prostate tumor. *Prostate* 2010;**70**:1066–1073.

58 Gao H, Priebe W, Glod J, Banerjee D. Activation of signal transducers and activators of transcription 3 and focal adhesion kinase by stromal cell-derived factor 1 is required for migration of human mesenchymal stem cells in response to tumor cell-conditioned medium. *Stem Cells* 2009;**27**:857–865.

59 Ho IA, Chan KY, Ng WH, *et al*. Matrix metalloproteinase 1 is necessary for the migration of human bone marrow-derived mesenchymal stem cells toward human glioma. *Stem Cells* 2009;**27**:1366–1375.

60 Dwyer RM, Potter-Beirne SM, Harrington KA, *et al*. Monocyte chemotactic protein-1 secreted by primary breast tumors stimulates migration of mesenchymal stem cells. *Clin Cancer Res* 2007;**13**:5020–5027.

61 Coffelt SB, Marini FC, Watson K, *et al*. The pro-inflammatory peptide LL-37 promotes ovarian tumor progression through recruitment of multipotent mesenchymal stromal cells. *Proc Natl Acad Sci U S A* 2009;**106**:3806–3811.

62 Shu HB, Agranoff AB, Nabel EG, *et al*. Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF-κB subunits in endothelial and epithelial cells. *Mol Cell Biol* 1993;**13**:6283–6289.

63 Collins T, Read MA, Neish AS, *et al*. Transcriptional regulation of endothelial cell adhesion molecules: NF-κB and cytokine-inducible enhancers. *FASEB J* 1995;**9**:899–909.

64 López-Franco O, Hernández-Vargas P, Ortiz-Muñoz G, *et al*. Parthenolide modulates the NF-κB-mediated inflammatory responses in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol* 2006;**26**:1864–1870.

65 Saadane A, Masters S, DiDonato J, *et al*. Parthenolide inhibits IκB kinase, NF-κB activation, and inflammatory response in cystic fibrosis cells and mice. *Am J Respir Cell Mol Biol* 2007;**36**:728–736.

66 Marui N, Offermann MK, Swerlick R, *et al*. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through an antioxidant-sensitive mechanism in human vascular endothelial cells. *J Clin Invest* 1993;**92**:1866–1874.

67 Brigelius-Flohé R, Friedrichs B, Maurer S, *et al*. Interleukin-1-induced nuclear factor kappa B activation is inhibited by overexpression of phospholipid hydroperoxide glutathione peroxidase in a human endothelial cell line. *Biochem J* 1997;**328**(Pt 1):199–203.

68 Jaruga B, Hong F, Kim WH, Gao B. IFN-γ/STAT1 acts as a proinflammatory signal in T cell-mediated hepatitis via induction of multiple chemokines and adhesion molecules: a critical role of IRF-1. *Am J Physiol Gastrointest Liver Physiol* 2004;**287**: G1044–G1052.

69 Aggarwal BB, Shishodia S, Sandur SK, *et al*. Inflammation and cancer: how hot is the link? *Biochem Pharmacol* 2006;**72**:1605–1621.

70 Balkwill F. Tumour necrosis factor and cancer. *Nat Rev Cancer* 2009;**9**:361–371.

71 Wu Y, Zhou BP. TNF-α/NF-κ/Snail pathway in cancer cell migration and invasion. *Br J Cancer* 2010;**102**:639–644.

72 Balkwill F. TNF-α in promotion and progression of cancer. *Cancer Metastasis Rev* 2006;**25**:409–416.

73 Ferrajoli A, Keating MJ, Manshouri T, *et al*. The clinical significance of tumor necrosis factor-α plasma level in patients having chronic lymphocytic leukemia. *Blood* 2002;**100**:1215–1219.

74 Ahmed MI, Salahy EE, Fayed ST, *et al*. Human papillomavirus infection among Egyptian females with cervical carcinoma: relationship to spontaneous apoptosis and TNF-α. *Clin Biochem* 2001;**34**:491–498.

75 Szlosarek P, Charles KA, Balkwill FR. Tumour necrosis factor-α as a tumour promoter. *Eur J Cancer* 2006;**42**:745–750.

76 Shake JG, Gruber PJ, Baumgartner WA, *et al*. Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* 2002;**73**:1919–1925.

77 Pittenger MF, Martin BJ. Mesenchymal stem cells and their potential as cardiac therapeutics. *Circ Res* 2004;**95**:9–20.

78 Levine JE. Implications of TNF-α in the pathogenesis and management of GVHD. *Acute Card Care* 2010;**12**:115–118.

79 Milano A, Iaffaioli RV, Caponigro F. The proteasome: a worthwhile target for the treatment of solid tumours? *Eur J Cancer* 2007;**43**:1125–1133.

80 Aaltonen KJ, Virkki LM, Malmivaara A, *et al*. Systematic review and meta-analysis of the efficacy and safety of existing TNF blocking agents in treatment of rheumatoid arthritis. *PLoS ONE* 2012;**7**: e30275.

81 Mann DL. Inflammatory mediators and the failing heart: past, present, and the foreseeable future. *Circ Res* 2002;**91**:988–998.

SECTION VIII

# The present and the future

CHAPTER 63

# Breaking news

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## 63.1 *In vitro* laboratory studies

Note, some reports in this section include both *in vitro* and *in vivo* studies.

- **Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern.** Matigian *et al.* [1].
- **CDH1 and IL1-beta expression dictates FAK and MAPKK-dependent cross-talk between cancer cells and human mesenchymal stem cells.** Al-toub *et al.* [2].
- **Electrical stimulation of human mesenchymal stem cells on conductive nanofibers enhances their differentiation toward osteogenic outcomes.** Hardy *et al.* [3]. The preparation of conductive fiber-based bone tissue scaffolds (nonwoven mats of electrospun polycaprolactone with an interpenetrating network of polypyrrole and polystyrenesulfonate) that enabled the electrical stimulation of human mesenchymal stem cells to enhance their differentiation toward osteogenic outcomes was described.
- **Genome-wide microRNA and gene analysis of mesenchymal stem cell chondrogenesis identifies an essential role and multiple targets for miR-140-5p.** Barter *et al.* [4].
- **Functional restoration of amyotrophic lateral sclerosis patient-derived mesenchymal stromal cells through inhibition of DNA methyltransferase.** Oh *et al.* [5] examined the correlation between excessive DNA methyltransferase expression and functional decline in mesenchymal stromal cells isolated from amyotrophic lateral sclerosis (ALS) patients.
- **Adipose-derived mesenchymal stem cells promote cell proliferation and invasion of epithelial ovarian cancer.** Chu *et al.* [6] investigated the impact of human adipose-derived mesenchymal stem cells derived from the omentum of normal donors on human epithelial ovarian cancer cells *in vitro* and *in vivo*.
- **Cultured human adipose tissue pericytes and mesenchymal stromal cells display a very similar gene expression profile.** Da Silva Meirelles *et al.* [7] found that both mesenchymal stromal cells and cultured pericytes were able to differentiate along mesenchymal pathways *in vitro*, displayed an essentially identical surface immunophenotype and exhibited the ability to suppress $CD3^+$ lymphocyte proliferation *in vitro*.
- **Pre-stimulation with FGF-2 increases *in vitro* functional coupling of mesenchymal stem cells with cardiac cells.** Preda *et al.* [8] investigated how pretreatment with growth factors modulates mesenchymal stem cell ability to integrate into the host tissue after transplantation, with particular focus on the expression of Cx43 and its cellular distribution.
- **Interleukin-17A-induced human mesenchymal stem cells are superior modulators of immunological function.** Sivanathan *et al.* [9] modified human bone-marrow-derived mesenchymal stem cells with interleukin (IL)-17A to enhance T cell immunosuppression but not their immunogenicity.
- **Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species.** Baglio *et al.* [10] characterized the full small RNAome of mesenchymal stem cell-produced exosomes. They set up a protocol for isolating exosomes released by early passage adipose- and bone marrow-derived MSCs and characterized them by electron microscopy, protein analysis and small RNA-sequencing.

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- **ATF4 licenses C/EBPβ activity in human mesenchymal stem cells primed for adipogenesis.** Cohen *et al.* [11] demonstrated that C/EBPβ bridges the transcriptional programs in naive, confluent cells and early differentiating pre-adipocytes. Moreover, they suggested that homo- and heterodimer formation poise C/EBPβ to execute diverse and stage-specific transcriptional programs by exploiting an expanded motif repertoire.
- **hMSC-derived VEGF release triggers the chemoattraction of alveolar osteoblasts.** Proksch *et al.* [12] identified secreted osteoprotegerin and vascular endothelial growth factor (VEGF) as potential chemoattractants of human MSC-directed osteoblast chemotaxis.
- **Altered mesenchymal niche cells impede generation of normal hematopoietic progenitor cells in leukemic bone marrow.** Lim *et al.* [13] focused on murine bone mesenchymal stem cells and their supporting function for normal hematopoietic cells in Notch1-induced acute T cell lymphocytic leukemia (T-ALL) mice. They demonstrated that the proliferative capability and differentiation potential of T-ALL mesenchymal stem cells were impaired due to accelerated cellular senescence. RNA-seq analysis revealed significant transcriptional alterations in leukemic mesenchymal stem cells. Osteoprotegerin was identified as a cytokine able to improve the function of T-ALL mesenchymal stem cells and to enhance normal hematopoietic progenitor cell output via the p38/ERK pathway.
- **Bone marrow-derived mesenchymal stromal cells differ in their attachment to fibronectin-derived peptides from term placenta-derived mesenchymal stromal cells.** Maerz *et al.* [14].
- **A molecular classification of human mesenchymal stromal cells.** Rohart *et al.* [15] addressed the question of a molecular classification of mesenchymal stromal cells with a large integrative analysis of many public mesenchymal stromal cell datasets. They derived a sparse classifier (the Rohart mesenchymal stromal cell test) that accurately distinguishes mesenchymal stromal cell from non-mesenchymal stromal cell samples with >97% accuracy on an internal training set of 635 samples from 41 studies derived on 10 different microarray platforms. The classifier was validated on an external test set of 1291 samples from 65 studies derived on 15 different platforms, with >95% accuracy. The genes that contribute to the mesenchymal stromal cell classifier formed a protein-interaction network that included known mesenchymal stromal cell markers. Further evidence of the relevance of this new mesenchymal stromal cell panel came from the high number of Mendelian disorders associated with mutations in more than 65% of the network. These result in mesenchymal defects, particularly impacting on skeletal growth and function. The Rohart mesenchymal stromal cell test is a simple *in silico* test that accurately discriminates mesenchymal stromal cells from fibroblasts, other adult stem/progenitor cell types or differentiated stromal cells. It has been implemented in the www.stemformatics.org resource, to assist researchers wishing to benchmark their own mesenchymal stromal cell datasets or data from the public domain. The code is available from the CRAN repository and all data used to generate the mesenchymal stromal cell test are available to download via the Gene Expression Omnibus or the Stemformatics resource.
- **Mesenchymal stromal cells induce peculiar alternatively activated macrophages capable of dampening both innate and adaptive immune responses.** Chiossone *et al.* [16] analyzed the functional outcome of the interactions between mesenchymal stromal cells and monocytes/macrophages. They showed that mesenchymal stromal cells supported the survival of monocytes that underwent differentiation into macrophages, in the presence of macrophage colony-stimulating factor. However, mesenchymal stromal cells skewed their polarization toward a peculiar M2-like functional phenotype ($M^{MSC}$), through a prostaglandin $E_2$ ($PGE_2$)-dependent mechanism. $M^{MSC}$ were characterized by high expression of scavenger receptors, increased phagocytic capacity, and high production of IL-10 and transforming growth factor (TGF)-β. These cytokines contributed to the immunoregulatory properties of $M^{MSC}$, which differed from those of typical IL-4-induced macrophages (M2).
- **Vav1 regulates mesenchymal stem cell differentiation decision between adipocyte and chondrocyte via Sirt1.** Qu *et al.* [17] found that Vav1, a guanine nucleotide exchange factor for Rho GTPase, was highly expressed in mesenchymal stem cells. Loss of Vav1 in mesenchymal stem cells led to spontaneous adipogenic but impaired chondrogenic differentiation, and accordingly Vav1 null mice displayed an increase in fat content and a decrease in cartilage. Conversely, ectopic expression of Vav1 in mesenchymal stem cells reversed this phenotype, and led to enhanced MSC differentiation into chondrocyte but retarded adipogenesis. Mechanistically, loss of Vav1 reduced the level of Sirt1, which was responsible for an increase of acetylated peroxisome proliferator-activated receptor-γ. As acetylation activates peroxisome proliferator-activated receptor-γ, it increased C/EBPα expression and promoted adipogenesis. On the other hand, loss of Vav1 resulted in an increase of acetylated Sox9, a target of Sirt1.
- **Mast cells promote proliferation and migration and inhibit differentiation of mesenchymal stem cells through PDGF.** Nazari *et al.* [18] found that mast cells promote mesenchymal stem cell proliferation and migration by suppressing their myogenic differentiation. Mast cells accomplish this via activation of the platelet-derived growth factor pathway, down-regulation of miR-145/143, and modulation of the myocardin–Klf4 axis. The findings in this study suggest a potential role for mesenchymal stem cell–mast cell interaction in the infarcted heart where mast cells may inhibit mesenchymal stem cells from differentiation and promote their proliferation whereby increased cardiac mesenchymal stem cell accumulation promotes eventual cardiac regeneration after mast cells cease activity.
- **Growth factors cross-linked to collagen microcarriers promote expansion and chondrogenic differentiation of human**

**mesenchymal stem cells.** Bertolo *et al.* [19]. In order to enhance cell attachment and produce a cost-effective cell culture solution with local stimulation of cells, basic fibroblast growth factor (bFGF) or TGF-β1 were covalently immobilized on microcarriers either by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide/*N*-hydroxysuccinimide (EDC/NHS) or riboflavin/ultraviolet (RB/UV) light-mediated crosslinking. Collagen microcarriers cross-linked with bFGF or TGF-β1 were used for expansion and chondrogenic differentiation of human mesenchymal stem cells. Crosslinking strengthened the collagen structure of the microcarriers and reduced collagenase-mediated degradation. Mesenchymal stem cells proliferated effectively on microcarriers cross-linked with bFGF, especially by EDC/NHS crosslinking. Chondrogenic differentiation of mesenchymal stem cells was induced by TGF-β1 cross-linked on microcarriers, promoting gene expression and protein accumulation of aggrecan, collagen type I and type II, as well as proteoglycans. Crosslinking by RB/UV enhanced chondrogenesis more than any other group. In addition, crosslinking reduced scaffold shrinkage exerted by mesenchymal stem cells during chondrogenesis, a desirable feature for microcarriers if used as a tissue defect filler.

- **Clusterin/Akt up-regulation is critical for GATA-4 mediated cytoprotection of mesenchymal stem cells against ischemia injury.** Yu *et al.* [20] found that clusterin expression was upregulated in mesenchymal stem cells overexpressing GATA-4 ($MSC^{GATA\text{-}4}$) compared with control mesenchymal stem cells transfected with empty-vector ($MSC^{Null}$). $MSC^{GATA\text{-}4}$ were tolerant to 72 h of hypoxia exposure, as shown by reduced lactate dehydrogenase release and higher uptake of the tetrazolium compound MTs. This protection was abrogated by transfecting Clu-siRNA into $MSC^{GATA\text{-}4}$. Second, they found that exogenous clusterin significantly decreased lactate dehydrogenase release and increased mesenchymal stem cell survival in a hypoxic environment. Moreover, mitochondrial membrane potential was maintained and caspase 3/7 activity was reduced by clusterin in a concentration-dependent manner. Third, they found that p-Akt expression in mesenchymal stem cells was upregulated following pretreatment with clusterin, with no change in total Akt. Moreover, cytoprotection mediated by clusterin was partially abrogated by the Akt inhibitor LY294002.
- **Three-dimensional bioprinting of thick vascularized tissues.** Kolesky *et al.* [21] describe a multimaterial three-dimensional (3D) bioprinting method that enables the creation of thick human tissues (>1 cm) replete with an engineered extracellular matrix, embedded vasculature, and multiple cell types. These 3D vascularized tissues could be actively perfused with growth factors for long durations (>6 weeks) to promote differentiation of human mesenchymal stem cells toward an osteogenic lineage *in situ*. The ability to construct and perfuse 3D tissues that integrate parenchyma, stroma, and endothelium is a major step toward creating human tissues for *ex vivo* and *in vivo* applications.
- **MiR-708 promotes steroid-induced osteonecrosis of femoral head, suppresses osteogenic differentiation by targeting SMAD3.** Hao *et al.* [22] made the hypothesis that corticosteroid-induced osteonecrosis of the femoral head (ONFH), a serious complication of corticosteroid use, was related to the differential expression of miRs in the mesenchymal stem cells of patients with ONFH. Mesenchymal stem cells were collected from the bone marrow of patients with ONFH. Samples were assigned to either those in a group that had received corticosteroids or those who had not (control group) in a 1 : 1 ratio. They used microRNA microarray analysis and real-time polymerase chain reaction to identify differentially expressed miRs. They also induced normal mesenchymal stem cells with corticosteroids to verify the differential expression. Subsequently, they selected some of the miRs for further studies, including microRNA target and pathway prediction, and functional analysis. They discovered that miR-708 was upregulated in ONFH patients and corticosteroid-treated mesenchymal stem cells. SMAD3 was identified as a direct target gene of miR-708, and functional analysis demonstrated that miR-708 could markedly suppress osteogenic differentiation and adipogenesis differentiation of mesenchymal stem cells. Inhibition of miR-708 rescued the suppressive effect of glucocorticoid on osteonecrosis. Therefore, they concluded that corticosteroid use results in overexpression of miR-708 in mesenchymal stem cells, and therefore targeting miR-708 may serve as a novel therapeutic biomarker for the prevention and treatment of ONFH.
- **Epac activation regulates human mesenchymal stem cells migration and adhesion.** Yu *et al.* [23] studied the role of Exchange protein directly activated by cyclic adenosine monophosphate (Epac), a novel protein discovered in the cyclic adenosine monophosphate signaling pathway that may have a potential role in regulating cell adhesion and migration by triggering the downstream Rap family signaling cascades. Their study evaluated the role of Epac in the homing of human mesenchymal stem cells. They confirmed that human mesenchymal stem cells expressed functional Epac and its activation enhanced the migration and adhesion of human mesenchymal stem cells significantly. Epac activation was further found to contribute directly to the chemotactic responses induced by stromal-cell-derived factor-1 (SDF-1), a known chemokine in regulating human mesenchymal stem cell homing. These findings suggested that Epac is connected to the SDF-1 signaling cascades by promoting mesenchymal stem cell adhesion and migration.

## 63.2 Preclinical *in vivo* animal studies

Note, some reports in this section include both *in vitro* and *in vivo* studies.

- **Mesenchymal stromal cells prevent allostimulation *in vivo* and control checkpoints of Th1 priming: migration of**

**human DC to lymph nodes and NK cell activation.** Consentius *et al.* [24].

- **A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction.** Holan *et al.* [25].
- **Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow.** Yue *et al.* [26]. Skeletal stem cells that are the major source of osteoblasts and adipocytes in adult bone marrow express leptin receptor (LepR). To test whether LepR regulates skeletal stem cell function, the authors conditionally deleted Lepr from limb bone marrow stromal cells, but not from the axial skeleton or hypothalamic neurons, using Prx1-Cre. Prx1-Cre;Lepr$^{fl/fl}$ mice exhibited normal body mass and normal hematopoiesis. However, limb bones from Prx1-Cre;Lepr$^{fl/fl}$ mice exhibited increased osteogenesis, decreased adipogenesis, and accelerated fracture healing. Leptin increased adipogenesis and reduced osteogenesis by activating Jak2/Stat3 signaling in bone marrow stromal cells. A high-fat diet increased adipogenesis and reduced osteogenesis in limb bones from wild-type mice, but not from Prx1-Cre;Lepr$^{fl/fl}$ mice. This reflected local effects of LepR on osteogenesis and adipogenesis by bone marrow stromal cells and systemic effects on bone resorption. Leptin/LepR signaling regulates adipogenesis and osteogenesis by mesenchymal stromal cells in the bone marrow in response to diet and adiposity.
- **Useful properties of undifferentiated mesenchymal stromal cells and adipose tissue as the source in liver-regenerative therapy studied in an animal model of severe acute fulminant hepatitis.** Manzini *et al.* [27].
- **Human adipose-derived mesenchymal stem cells modulate experimental autoimmune arthritis by modifying early adaptive T cell responses.** Lopez-Santalla *et al.* [28].
- **A computer-designed scaffold for bone regeneration within cranial defect using human dental pulp stem cells.** Kwon *et al.* [29] used a computer-designed, solvent-free scaffold and human dental pulp stem cells (DPSCs) to regenerate new bone in cranial bone defects. They used solid freeform fabrication with biodegradable polyesters (MPEG–(PLLA–co-PGA–co-PCL) (PLGC)) to fabricate a computer-designed scaffold. The solid freeform fabrication technology gave quick and reproducible results. To assess bone tissue engineering *in vivo*, the computer-designed, circular PLGC scaffold was implanted into full-thickness cranial bone defects and monitored by microcomputed tomography and histology of the *in vivo* tissue-engineered bone. New bone formation of more than 50% was observed by micro-computed tomography and histology only with the use of the PLGC scaffold with human DPSCs and osteogenic factors. The PLGC scaffold gradually degraded, as evidenced by the fluorescent-labeled PLGC scaffold, which provided information to track biodegradation of the implanted PLGC scaffold.
- **Mesenchymal stem cell expression of SDF-1β synergizes with BMP-2 to augment cell-mediated healing of critical-sized mouse calvarial defects.** Herberg *et al.* [30] suggested that SDF-1β provides synergistic effects supporting BMP-2-induced, BMSC-mediated bone formation and appears suitable for optimization of bone augmentation in combination therapy protocols *in vivo*.
- **Mouse white adipose tissue-derived mesenchymal stem cells gain pericentral and periportal hepatocyte features after differentiation *in vitro*, which are preserved *in vivo* after hepatic transplantation.** Winkler *et al.* [31]. Adipose tissue-derived mesenchymal stem cells were isolated from immunodeficient C57BL/6 (B6.129S6-Rag2tm1FwaPrf1tm1Clrk) mice and differentiated into the hepatocytic phenotype by applying a simple protocol. Their physiological and metabolic functions were analyzed *in vitro* and after hepatic transplantation *in vivo*. Mesenchymal stem cells changed their morphology from a fibroblastoid into shapes of osteocytes, chondrocytes, adipocytes, and hepatocytes. Typical for mesenchymal stem cells, hematopoietic marker genes were not expressed. CD90, which is not expressed on mature hepatocytes, decreased significantly after hepatocytic differentiation. Markers indicative for liver development (like hepatic nuclear factor 4 alpha) or for perivenous hepatocyte specification (like cytochrome P450 subtype 3a11) and CD26 were significantly elevated. Periportal hepatocyte-specific markers (like carbamoylphosphate synthetase-1, the entry enzyme of the urea cycle) were upregulated. Consequently, cytochrome P450 enzyme activity and urea synthesis increased significantly to values comparable to cultured primary hepatocytes. Both perivenous and periportal qualities were preserved after hepatic transplantation and integration into the host parenchyma.
- **Differential expression of adhesion-related proteins and MAPK pathways leads to suitable osteoblast differentiation of human mesenchymal stem cell subpopulations.** Leyva-Leyva *et al.* [32] analyzed the expression profiles of adhesion-related proteins during osteoblastic differentiation of two human MSCs subpopulations ($CD105^+$ and $CD105^-$) and proposed a strategy for assembling bone grafts based on its adhesion ability. *In vitro* experiments of osteogenic differentiation in $CD105^-$ cells showed superior adhesion efficiency and twofold increase of α-actinin expression compared with $CD105^+$ cells at the maturation stage. Levels of activated β1-integrin increased in $CD105^-$ cells during the process. Additionally, the $CD105^-$ subpopulation showed a threefold increase of phosphorylated FAKY397 compared with $CD105^+$ cells. An *in vivo* trial demonstrated that grafts containing $CD105^-$ cells showed osteocytes embedded in a mineralized matrix, promoted adequate graft integration, increased host vascular infiltration, and produced efficient intramembranous repair. In contrast, grafts containing $CD105^+$ cells showed deficient endochondral ossification and fibrocartilaginous tissue.
- **Adipose-derived mesenchymal stem cells ameliorate ulcerative colitis through miR-1236 negatively regulating the expression of retinoid-related orphan receptor gamma.** Zhang *et al.* [33] investigated the effect of adipose-derived

mesenchymal stem cells (ASCs) on 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ulcerative colitis in rat colon tissues. Qualitative real-time polymerase chain reaction and immunofluorescence analyses were performed to monitor the expression of miR-1236 and its target molecule, retinoid-related orphan receptor γ (RORγ). Regulation of the expression of RORγ by miR-1236 was assessed using luciferase reporter construct assays and miR-1236 mimic transfection. The relationship between miR-1236 and RORγ was further investigated in HT29 cells induced by tumor necrosis factor (TNF)-α. ASCs highly ameliorated ulcerative colitis and decreased the inflammation markers in rats with TNBS-induced ulcerative colitis. In addition, ASCs upregulated the expression of miR-1236 and decreased the expression of RORγ in the TNBS-induced rat model of ulcerative colitis. The luciferase reporter assay and bioinformatic analysis demonstrated that the expression of RORγ was directly targeted and regulated by miR-1236. Specifically, the expression of RORγ was suppressed by miR-1236 mimic and enhanced by miR-1236 inhibitor. It was demonstrated that exogenous miR-1236 mimic could inhibit the expression of RORγ in HT29 cell induced by TNF-α. ASCs effectively alleviated ulcerative colitis in rats with the expression of miR-1236 alteration, and miR-1236 may play important roles in ulcerative colitis by down-regulating the expression of RORγ.

- **Cartilage repair using composites of human umbilical cord blood-derived mesenchymal stem cells and hyaluronic acid hydrogel in a minipig model.** Ha *et al.* [34] confirmed the consistent cartilage regeneration potential of human umbilical cord blood-derived mesenchymal stem cells in a pig model using three different cell lines. The cellular characteristics of the human umbilical cord blood-derived mesenchymal stem cells influenced the degree of cartilage regeneration potential.
- **Mesenchymal stem cells regulate melanoma cancer cells extravasation to bone and liver at their perivascular niche.** Correa *et al.* [35] reported that local mesenchymal stem cells, acting as pericytes, regulate the extravasation of melanoma cells specifically to murine bone marrow and liver, the two commonest sites for melanoma metastases.
- **Bone marrow-derived mesenchymal stem cells co-expressing interleukin-18 and interferon-β exhibit potent antitumor effect against intracranial glioma in rats.** Xu *et al.* [36]. Bone-marrow-derived MSCs were genetically engineered to express IL-18 and interferon (IFN)-β by transfection of recombinant lentivirus-mediated gene transfer. These cells significantly prolonged the survival and inhibited tumor growth in a rat intracranial glioma model.
- **Human mesenchymal stem cells genetically engineered to overexpress brain-derived neurotrophic factor improve outcomes in Huntington's disease mouse models.** Double-blinded studies were performed by Pollock *et al.* [37] to examine the effects of intrastriatally transplanted human mesenchymal stem cell/brain-derived neurotrophic factor (BDNF) on disease progression in two strains of immune-suppressed HD transgenic mice: YAC128 and R6/2. Mesenchymal stem cell/BDNF treatment decreased striatal atrophy in YAC128 mice. Mesenchymal stem cell/BDNF treatment also significantly reduced anxiety as measured in the open-field assay. Both mesenchymal stem cell and mesenchymal stem cell/BDNF treatments induced a significant increase in neurogenesis-like activity in R6/2 mice. Mesenchymal stem cell/BDNF treatment also increased the mean lifespan of the R6/2 mice.
- **Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration.** Because toll-like receptors and IL-1R1 have been shown to influence the repair process of various tissues, Martino *et al.* [38] explored their role during bone regeneration, seeking to design regenerative strategies integrating a control of their signaling. They showed that IL-1R1/MyD88 signaling negatively regulates bone regeneration in the mouse. Furthermore, IL-1β, which is released at the bone injury site, inhibited the regenerative capacities of mesenchymal stem cells. Mechanistically, IL-1R1/MyD88 signaling impaired mesenchymal stem cell proliferation, migration, and differentiation by inhibiting the Akt/GSK-3β/β-catenin pathway. Lastly, as a proof of concept, they engineered a mesenchymal stem cell delivery system integrating inhibitors of IL-1R1/MyD88 signaling. Using this strategy, they considerably improved mesenchymal-stem-cell-based bone regeneration in the mouse, demonstrating that this approach may be useful in regenerative medicine applications.
- **Systemic mesenchymal stromal cell transplantation prevents functional bone loss in a mouse model of age-related osteoporosis.** Kiernan *et al.* [39], using minimally expanded exogenous mesenchymal stromal cells injected systemically into a mouse model of human age-related osteoporosis, showed long-term engraftment and markedly increased bone formation. This led to improved bone quality and turnover and, importantly, sustained microarchitectural competence. These data establish proof of concept that mesenchymal stromal cell transplantation may be used to prevent or treat human age-related osteoporosis.
- **Intra-articular injection of synovial mesenchymal stem cells improves cartilage repair in a mouse injury model.** Mak *et al.* [40] isolated a Sca-1 positive, chondrogenesis-capable population of mouse synovial mesenchymal stem cells from C57BL/6 and MRL/MpJ "super-healer" strains. Intra-articular injection of $Sca1^+$ green fluorescent $protein^+$ synovial cells from C57BL/6 or MRL/MpJ into C57BL/6 mice following cartilage injury led to increased cartilage repair by 4 weeks after injury. Green fluorescent protein expression was detected in the injury site at 2 weeks, but not 4 weeks after injury. These results suggest that synovial stem/progenitor cells, regardless of strain background, have beneficial effects when injected into an injured joint.
- **Delivery of mesenchymal stem cells in biomimetic engineered scaffolds promotes healing of diabetic ulcers.** Assi *et al.* [41]. Rolled scaffolds containing mesenchymal stem cells

were hypoxic and induced mesenchymal stem cell synthesis and secretion of VEGF. Diabetic mice with wounds treated with rolled scaffolds containing mesenchymal stem cells showed increased healing compared with controls. Histologic examination showed increased cellular proliferation, increased VEGF expression and capillary density, and increased numbers of macrophages, fibroblasts, and smooth muscle cells. Addition of laminin to the collagen scaffold enhanced these effects

- **Human mesenchymal stromal cells reduce influenza A H5N1-associated acute lung injury *in vitro* and *in vivo*.** Chan *et al.* [42] found that, *in vitro*, influenza A/H5N1 infection impaired alveolar fluid clearance more than did seasonal virus, mimicking its greater severity in patients. They demonstrated that this impairment was mediated by the release of soluble factors from infected cells, leading to downregulation of alveolar sodium and chloride transporters. Mesenchymal stromal cells prevented or reduced this effect *in vitro* and *in vivo* in A/H5N1-infected mice. These cells thus provide a potentially effective treatment for acute lung injury in severe influenza.
- **A novel therapeutic approach using mesenchymal stem cells to protect against *Mycobacterium abscessus*.** Kim *et al.* [43] explored the use of mesenchymal stem cells as a novel therapeutic strategy against *Mycobacterium abscessus*, which is the most drug-resistant and difficult-to-treat mycobacterial pathogen. The systemic intravenous injection of mesenchymal stem cells not only improved mouse survival but also enhanced bacterial clearance in the lungs and spleen. Additionally, mesenchymal stem cells enhanced IFN-γ, TNF-α, IL-6, monocyte chemoattractant protein-1, nitric oxide (NO), and $PGE_2$ production and facilitated $CD4^+/CD8^+$ T cell, $CD11b^{high}$ macrophage and monocyte recruitment in the lungs of *M. abscessus*-infected mice. To precisely elucidate the functions of mesenchymal stem cells in *M. abscessus* infection, an *in vitro* macrophage infection system was used. Mesenchymal stem cells caused markedly increased NO production via nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation in *M. abscessus*-infected macrophages cultured in the presence of IFN-γ. Inhibiting NO or NF-κB signaling using specific inhibitors reduced the antimycobacterial activity of mesenchymal stem cells. Furthermore, the cellular crosstalk between TNF-α released from IFN-γ-stimulated *M. abscessus*-infected macrophages and $PGE_2$ produced by mesenchymal stem cells was necessary for the mycobacterial-killing activity of the macrophages. Finally, the importance of increased NO production in response to mesenchymal stem cell administration was confirmed in the mouse *M. abscessus* infection model. These results suggest that mesenchymal stem cells may offer a novel therapeutic strategy for treating this drug-resistant mycobacterial infection by enhancing the bacterial-killing power of macrophages.
- **Human amniotic mesenchymal stem cells alleviate lung injury induced by ischemia and reperfusion after cardiopulmonary bypass in dogs.** Qiang *et al.* [44] considered that transplantation of mesenchymal stem cells may inhibit pathological immune processes contributing to ischemia–reperfusion (I–R) injury in the lung. This study aimed to assess the capacity of human amniotic mesenchymal stem cells (hAMSCs) to ameliorate I–R injury in a dog model of cardiopulmonary bypass (CPB). Dissociated hAMSCs were cultured *ex vivo* and their immunophenotypes were assessed by flow cytometry and immunohistochemistry. A dog model of CPB was established by surgical blockage of the aorta for 1 h. Dogs either underwent mock surgery (sham group), CPB (model group), or CPB followed by the femoral injection of $2 \times 10^7$ hAMSCs ($n = 6$). Anti-human nuclei staining revealed hAMSCs in the lungs 3 h after surgery. The oxygen index (OI) and respiratory index (RI) of arterial blood were measured using a biochemical analyzer. Venous blood TNF-α, IL-8, matrix metallopeptidase (MMP)-9, and IL-10 concentrations were measured by enzyme-linked immunosorbent assay. Pathological changes in the lung were assessed by light microscopy. Third-generation cultured hAMSCs expressed high levels of CD29, CD44, CD49D, CD73, and CD166 levels, but low CD34 or CD45 amounts and their cytoplasm contained vimentin. In CPB model animals the OI was elevated and the RI was reduced. TNF-α, IL-8, and MMP-9 levels were elevated, and IL-10 levels reduced within 3 h ($P < 0.05$), but hAMSC transplantation significantly ameliorated these changes ($P < 0.05$). Pathological changes observed in the hAMSC group were significantly less severe than those in the CPB group. The authors concluded that hAMSC transplantation can downregulate proinflammatory factors and reduce MMP-9 levels, while upregulating the anti-inflammatory molecule IL-10, thus reducing I–R lung injury in this dog model of CPB.
- **Allogeneic mesenchymal stem cells in combination with hyaluronic acid for the treatment of osteoarthritis in rabbits.** Chiang *et al.* [45]. The purpose of this study was to investigate the effects of the intra-articular injection of allogeneic mesenchymal stem cells in an *in vivo* anterior cruciate ligament transection (ACLT) model of osteoarthritis in rabbits. Allogeneic bone-marrow-derived mesenchymal stem cells were isolated and cultured under hypoxic conditions (1% $O_2$). Eight weeks after ACLT, MSCs suspended in hyaluronic acid (HA) were injected into the knees while the contralateral knees were injected with HA alone. Additional controls consisted of a sham operation group as well as an untreated osteoarthritis group. The tissues were analyzed by macroscopic examination as well as histologic and immunohistochemical methods at 6 and 12 weeks after transplantation. At 6 and 12 weeks the joint surface showed less cartilage loss and surface abrasion after mesenchymal stem cell injection compared with the tissues receiving HA injection alone. Significantly better histological scores and cartilage content were observed with mesenchymal stem cell transplantation. Furthermore, engraftment of allogenic mesenchymal stem cells was evident in surface cartilage. The authors concluded

that injection of allogeneic mesenchymal stem cells reduced the progression of osteoarthritis *in vivo*.

- **Quantitative evaluation of human bone mesenchymal stem cells rescuing fulminant hepatic failure in pigs.** Shi *et al.* [46] used an in-house large animal (pig) model of rescue of fulminant hepatic failure (FHF) by human bone marrow mesenchymal stem cells and profiled the cells' activities. The control and transplantation groups of pigs ($n = 15$ per group) both received a D-galactosamine (D-Gal) injection (1.5 g/kg). The transplantation group received mesenchymal stem cells via intraportal vein infusion ($3 \times 10^6$ cells/kg) immediately after D-Gal administration. The stem cell recipient interactions were quantitatively evaluated by biochemical function, cytokine array, metabolite profiling, transcriptome sequencing, and immunohistochemistry. All pigs in the control group died within an average of 3.22 days, whereas 13/15 pigs in the transplantation group lived >14 days. The cytokine array and metabolite profiling analyses revealed that mesenchymal stem cell transplantation suppressed D-Gal-induced life-threatening cytokine storms and stabilized FHF within 7 days, while human-derived hepatocytes constituted only ~4.5% of the pig hepatocytes. The functional synergy analysis of the observed profile changes indicated that the implanted mesenchymal stem cells altered the pigs' cytokine responses to damage through paracrine effects. Delta-like ligand 4 was validated to assist liver restoration in both pig and rat FHF models.
- **Mesenchymal stromal cell–mediated neuroprotection and functional preservation of retinal ganglion cells in a rodent model of glaucoma.** Mead *et al.* [47] stated that glaucoma is a leading cause of irreversible blindness involving loss of retinal ganglion cells (RGCs), that mesenchymal stromal cells have shown promise as a paracrine-mediated therapy for compromised neurons but that it is unknown whether dental pulp stem cells (DPSCs) are effective as a cellular therapy in glaucoma and how their hypothesized influence compares with other more widely researched mesenchymal stromal cell sources. Their study aimed to compare the efficacy of adipose-derived stem cells, bone-marrow-derived mesenchymal stromal cells (BMSCs) and DPSCs in preventing the loss of RGCs and visual function when transplanted into the vitreous of glaucomatous rodent eyes. Thirty-five days after induction of raised intraocular pressure followed by intravitreal mesenchymal stromal cell transplantation, $Brn3a^+$ RGC numbers, retinal nerve fiber layer thickness (RNFL), and RGC function were evaluated by immunohistochemistry, optical coherence tomography, and electroretinography respectively. Control glaucomatous eyes that were sham-treated with heat-killed DPSCs had a significant loss of RGC numbers, RNFL thickness, and function compared with intact eyes. BMSCs and, to a greater extent, DPSCs provided significant protection from RGC loss and RNFL thinning and preserved RGC function.
- **Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration.** Maruyama *et al.* [48] explored the biology and therapeutic aspects of the cells of the suture mesenchyme. The suture mesenchyme serves as a growth center for calvarial morphogenesis and has been postulated to act as the niche for skeletal stem cells. Aberrant gene regulation causes suture dysmorphogenesis, resulting in craniosynostosis, one of the most common craniofacial deformities. Owing to various limitations, especially the lack of suture stem cell isolation, reconstruction of large craniofacial bone defects remains highly challenging. In this article the authors provided the first evidence for an Axin2-expressing stem cell population with long-term self-renewing, clonal expanding, and differentiating abilities during calvarial development and homeostastic maintenance. These cells, which reside in the suture midline, were found to contribute directly to injury repair and skeletal regeneration in a cell autonomous fashion. These findings demonstrate the identity of these cells as skeletal stem cells with innate capacities to replace the damaged skeleton and should permit further elucidation of the biology of stem-cell-mediated craniofacial skeletogenesis, leading to the elucidation of the complex nature of congenital disease and the use of these cells in regenerative medicine.
- **Mesenchymal stem cells alleviate LPS-induced acute lung injury in mice by mir-142a-5p-controlled pulmonary endothelial cell autophagy.** Zhou and You [49] found that mesenchymal stem cell transplantation significantly reduced the severity of lipopolysaccharide (LPS)-induced acute lung injury in mice. Mesenchymal stem cells increased autophagy of pulmonary endothelial cells (PECs) to promote PEC survival. Mesenchymal stem cells increased beclin-1 protein but not mRNA. miR-142a-5p was found to target the 3′-UTR of beclin-1 mRNA to inhibit its protein translation in PECs. Mesenchymal stem cells reduced the levels of miR-142a-5p in PECs from LPS-treated mice. It was concluded that mesenchymal stem cells may alleviate LPS-induced acute lung injury through downregulation of miR-142a-5p, which allows PECs to increase beclin-1-mediated cell autophagy.
- **Transplantation of CXCR4 overexpressed mesenchymal stem cells augments regeneration in degenerated intervertebral discs.** *Wei et al.*'s [50] aim was to evaluate whether high expression of CXCR4 enhanced the migration of mesenchymal stem cells and increased the efficiency of intervertebral disc (IVD) regeneration. Mesenchymal stem cells overexpressing *CXCR* were created by lentiviral-*CXCR4*-overexpressing transfection, labeled with superparamagnetic iron oxide (SPIO), and transplanted into rabbit degenerative IVD induced by annulus puncture. X-ray and T2-weighted magnetic resonance images of the spine were obtained at 0, 8, and 16 weeks posttransplantation. The transplanted stem cells were traced by both magnetic resonance imaging and Prussian blue staining. The stem-cell-based IVD degeneration was evaluated by quantifying the expression of aggrecan and type II collagen. An *in vitro* chemotaxis test was performed to study the migration of CXCR4 mesenchymal stem cells to the supplement of SDF-1. The CXCR4-overexpressing mesenchymal stem cells stably

elevated the expression of CXCR4 and increased the migration to SDF-1. The SPIO-labeled CXCR4 mesenchymal stem cells could be detected within the IVD by magnetic resonance imaging up till 16 weeks after transplantation. Prussian blue staining showed more SPIO-positive cells within the IVD transplanted with CXCR4 mesenchymal stem cells. Compared with the control group, loss of disc height was slowed while the mRNA expression of aggrecan and type II collagen was increased by mesenchymal stem cell transplantation, especially in the IVD supplemented with CXCR4 mesenchymal stem cells. CXCR4 overexpression promoted mesenchymal stem cell retention within the IVD and enhanced the cell-based IVD regeneration.

- **Chromatographically isolated $CD63^+CD81^+$ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI.** Kim *et al.* [51] stated that extracellular vesicles (EVs) secreted by cells present an attractive strategy for developing new therapies, but progress in the field has been limited by several issues: the quality of the EVs varies with the type and physiological status of the producer cells, protocols used to isolate the EVs are difficult to scale up, and assays for efficacy are difficult to develop. They addressed these issues by using human mesenchymal stem/stromal cells that produce EVs when incubated in a protein-free medium, preselecting the preparations of mesenchymal stem/stromal cells with a biomarker for their potency in modulating inflammation, incubating the cells in a chemically defined protein-free medium that provided a stable environment, isolating the EVs with a scalable chromatographic procedure, and developing an *in vivo* assay for efficacy of the cells in suppressing neuroinflammation after traumatic brain injury in mice. They demonstrated that intravenous infusion of the isolated EVs shortly after induction of traumatic brain injury rescued pattern separation and spatial learning impairments 1 month later.

## 63.3 Clinical trials

When the term "mesenchymal stem cells" was searched in the ClinicalTrials.gov database on April 7, 2016, a total of 596 trials were listed. When the term "mesenchymal stromal cells" was searched in the ClinicalTrials.gov database on the same day a total of 123 trials were listed. In each search some trials were recruiting, some were completed, some were yet to be initiated, and some were withdrawn.

A relatively small number of clinical trials using mesenchymal stem/stromal cells have been published. Examples of trials that have been published for cardiac, neurological, pulmonary, or gastrointestinal indications are as follows.

- **A phase II dose-escalation study of allogeneic mesenchymal precursor cells in patients with ischemic or non-ischemic heart failure.** Perin *et al.* [52]. Transendocardial injections of allogeneic mesenchymal precursor cells were feasible and safe in chronic heart failure patients. High-dose allogeneic mesenchymal precursor cells may provide benefits in this population.
- **Safety and clinical effects of mesenchymal stem cells secreting neurotrophic factor transplantation in patients with amyotrophic lateral sclerosis: results of phase 1/2 and 2a clinical trials.** Petrou *et al.* [53] stated that clinical studies show neurotrophic growth factors (NTFs) extend the survival of motor neurons in ALS and that the combined delivery of these neurotrophic factors has a strong synergistic effect. They have developed a culture-based method for inducing mesenchymal stem cells to secrete neurotrophic factors. These mesenchymal stem-cell-NTF cells have been shown to be protective in several animal models of neurodegenerative diseases. The objective of this study was to determine the safety and possible clinical efficacy of autologous mesenchymal stem-cell-NTF cells transplantation in patients with ALS. In these open-label proof-of-concept studies, patients with ALS were enrolled between June 2011 and October 2014 at the Hadassah Medical Center in Jerusalem, Israel. All patients were followed up for 3 months before transplantation and 6 months after transplantation. In the phase 1/2 part of the trial, six patients with early-stage ALS received intramuscular (IM) injections and six patients with more advanced disease received intrathecal (IT) transplantation. In the second stage, a phase 2a dose-escalating study, 14 patients with early-stage ALS received a combined IM and IT transplantation of autologous mesenchymal stem-cell-NTF cells. All patients were administered a single dose of mesenchymal stem-cell-NTF cells. The primary end points of the studies were safety and tolerability of this cell therapy. Secondary end points included the effects of the treatment on various clinical parameters, such as the ALS Functional Rating Scale–Revised score and respiratory function. Among the 12 patients in the phase 1/2 trial and the 14 patients in the phase 2a trial aged 20 and 75 years, the treatment was found to be safe and well tolerated over the study follow-up period. Most of the adverse effects were mild and transient, without any treatment-related serious adverse event. The rate of progression of the forced vital capacity and of the ALS Functional Rating Scale–Revised score in the IT (or IT + IM)-treated patients was reduced (from −5.1% to −1.2%/month percentage predicted forced vital capacity, $P < 0.04$, and from −1.2 to 0.6 ALS Functional Rating Scale–Revised points/month, $P = 0.052$) during the 6 months following mesenchymal stem-cell-NTF cell transplantation versus the pretreatment period. Of these patients, 13 (87%) were defined as responders to either ALS Functional Rating Scale–Revised or forced vital capacity, having at least 25% improvement at 6 months after treatment in the slope of progression. The authors conclude that IT and IM administration of mesenchymal stem-cell-NTF cells in patients with ALS is safe and provide indications of possible clinical benefits, to be confirmed in upcoming clinical trials.

- **A placebo-controlled, randomized trial of mesenchymal stem cells in COPD.** Weiss *et al.* [54]. Sixty-two patients with chronic obstructive pulmonary disease (COPD) at six sites were randomized to double-blinded intravenous infusions of either allogeneic MSCs (Prochymal; Osiris Therapeutics Inc.) or vehicle control. Patients received four monthly infusions ($100 \times 10^6$ cells/infusion) and were subsequently followed for 2 years after the first infusion. End points included comprehensive safety evaluation, pulmonary function testing, and quality-of-life indicators, including questionnaires, 6 min walking test, and assessments of systemic inflammation. All study patients completed the full infusion protocol, and 74% completed the 2-year follow-up. There were no infusional toxicities and no deaths or serious adverse events deemed related to mesenchymal stem cell administration. There were no significant differences in the overall number of adverse events, frequency of COPD exacerbations, or worsening of disease in patients treated with mesenchymal stem cells. There were no significant differences in pulmonary function testings or quality-of-life indicators; however, an early, significant decrease in levels of circulating C-reactive protein was observed in patients treated with mesenchymal stem cells who had elevated C-reactive protein levels at study entry.
- **A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy.** Forbes *et al.* [55]. This was a phase 2, open-label, multicenter study that included 16 patients (21–55 years old; six men) with infliximab- or adalimumab-refractory, endoscopically confirmed, active luminal Crohn's disease (CD) (CD activity index CDAI > 250). Subjects were given intravenous infusions of allogeneic mesenchymal stromal cells ($2 \times 10^6$ cells/kg body weight) weekly for 4 weeks. The primary end point was clinical response (decrease in CDAI > 100 points) 42 days after the first mesenchymal stromal cell administration; secondary end points were clinical remission (CDAI < 150), endoscopic improvement (a CD endoscopic index of severity CDEIS < 3 or a decrease by >5), quality of life, level of C-reactive protein, and safety. Among the 15 patients who completed the study, the mean CDAI score was reduced from 370 (median, 327; range, 256–603) to 203 (median, 129) at day 42 ($P < 0.0001$). The mean CDAI scores decreased after each mesenchymal stromal cell infusion (370 before administration, 269 on day 7, 240 on day 14, 209 on day 21, 182 on day 28, and 203 on day 42). Twelve patients had a clinical response (80%; 95% confidence interval, 72–88%; mean reduction in CDAI, 211; range, 102–367), eight had clinical remission (53%; range, 43–64%; mean CDAI at day 42, 94; range, 44–130). Seven patients had endoscopic improvement (47%), for whom the mean CDEIS scores decreased from 21.5 (range, 3.3–33) to 11.0 (range, 0.3–18.5). One patient had a serious adverse event (two dysplasia-associated lesions), but these were not thought to be due to the mesenchymal stromal cells.

## 63.4 Regulatory approval for marketing mesenchymal stromal cell products

Mesoblast's licensee in Japan for acute graft-versus-host disease, JCR Pharmaceuticals Co. Ltd, has launched its mesenchymal stromal-cell-based product for children and adults with acute graft-versus-host disease: TEMCELL® HS Inj. is the first allogeneic cell-based medicine to receive full regulatory approval in Japan.

In May 2012 Osiris Therapeutics Inc. (now owned by Mesoblast) announced that it had received market authorization from Health Canada to market its mesenchymal stem cell therapy Prochymal® (remestemcel-L), for the treatment of acute graft-versus-host disease in children.

In June 2012 the same company announced that it had received consent from New Zealand to market Prochymal® (remestemcel-L) for the treatment of acute graft-versus-host disease.

Mesenchymal stromal cell products have been approved for other indications in South Korea. Hearticellgram-AMI (FCB-Pharmicell), an autologous mesenchymal stromal cell intracoronary injection for the treatment of acute myocardial infarction, was approved in South Korea in 2011. Cupistem (Anterogen) for anal fistula and Cartistem (Medipost) for cartilage injury and osteoarthritis were approved in January 2012 in South Korea. Cartistem is an allogeneic mesenchymal stromal cell product derived from human umbilical cord blood and Cupistem is an autologous mesenchymal stromal cell product.

## References


1 Matigian N, Brooke G, Zaibak F, *et al.* Multipotent human stromal cells isolated from cord blood, term placenta and adult bone marrow show distinct differences in gene expression pattern. *Genom Data* 2014;**3**:70–74.

2 Al-toub M, Vishnubalaji R, Hamam R, *et al.* CDH1 and IL1-beta expression dictates FAK and MAPKK-dependent cross-talk between cancer cells and human mesenchymal stem cells. *Stem Cell Res Ther* 2015;**6**:135.

3 Hardy JG, Villancio-Wolter MK, Sukhavasi RC, *et al.* Electrical stimulation of human mesenchymal stem cells on conductive nanofibers enhances their differentiation toward osteogenic outcomes. *Macromol Rapid Commun* 2015;**36**(21):1884–1890.

4 Barter MJ, Tselepi M, Gómez R, *et al.* Genome-wide microRNA and gene analysis of mesenchymal stem cell chondrogenesis identifies an essential role and multiple targets for miR-140-5p. *Stem Cells* 2015;**33**(11):3266–3280.

5 Oh YS, Kim SH, Cho GW. Functional restoration of amyotrophic lateral sclerosis patient-derived mesenchymal stromal cells through inhibition of DNA methyltransferase. *Cell Mol Neurobiol* 2015; in press. doi: 10.1007/s10571-015-0242-2.

6 Chu Y, Tang H, Guo Y, *et al.* Adipose-derived mesenchymal stem cells promote cell proliferation and invasion of epithelial ovarian cancer. *Exp Cell Res* 2015;**337**(1):16–27.

7 Da Silva Meirelles L, Malta TM, de Deus Wagatsuma VM, *et al.* Cultured human adipose tissue pericytes and mesenchymal stromal cells display a very similar gene expression profile. *Stem Cells Dev* 2015;**24**(23):2822–2840.

8 Preda MB, Rosca AM, Tutuianu R, Burlacu A. Pre-stimulation with FGF-2 increases *in vitro* functional coupling of mesenchymal stem cells with cardiac cells. *Biochem Biophys Res Commun* 2015;**464**(2): 667–673.

9 Sivanathan KN, Rojas-Canales DM, Hope CM, *et al.* Interleukin-17A-induced human mesenchymal stem cells are superior modulators of immunological function. *Stem Cells* 2015;**33**(9):2850–2863.

10 Baglio SR, Rooijers K, Koppers-Lalic D, *et al.* Human bone marrow- and adipose-mesenchymal stem cells secrete exosomes enriched in distinctive miRNA and tRNA species. *Stem Cell Res Ther* 2015;**6**:127.

11 Cohen DM, Won KJ, Nguyen N, *et al.* ATF4 licenses C/EBPβ activity in human mesenchymal stem cells primed for adipogenesis. *Elife* 2015;**4**:e06821.

12 Proksch S, Bittermann G, Vach K, *et al.* hMSC-derived VEGF release triggers the chemoattraction of alveolar osteoblasts. *Stem Cells* 2015;**33**(10):3114–3124.

13 Lim M, Pang Y, Ma S, *et al.* Altered mesenchymal niche cells impede generation of normal hematopoietic progenitor cells in leukemic bone marrow. *Leukemia* 2016;**30**(1):154–162.

14 Maerz JK, Roncoroni LP, Goldeck D, *et al.* Bone marrow-derived mesenchymal stromal cells differ in their attachment to fibronectin-derived peptides from term placenta-derived mesenchymal stromal cells. *Stem Cell Res Ther* 2016;**7**(1):29.

15 Rohart F, Mason EA, Matigian N, *et al.* A molecular classification of human mesenchymal stromal cells. *PeerJ* 2016;**4**:e1845.

16 Chiossone L, Conte R, Spaggiari GM, *et al.* Mesenchymal stromal cells induce peculiar alternatively activated macrophages capable of dampening both innate and adaptive immune responses. *Stem Cells* 2016; in press. doi: 10.1002/stem.2369.

17 Qu P, Wang L, Min Y, *et al.* Vav1 regulates mesenchymal stem cell differentiation decision between adipocyte and chondrocyte via Sirt1. *Stem Cells* 2016; in press. doi: 10.1002/stem.2365.

18 Nazari M, Ni NC, Lüdke A, *et al.* Mast cells promote proliferation and migration and inhibit differentiation of mesenchymal stem cells through PDGF. *J Mol Cell Cardiol* 2016;**94**:32–42.

19 Bertolo A, Arcolino F, Capossela S, *et al.* Growth factors cross-linked to collagen microcarriers promote expansion and chondrogenic differentiation of human mesenchymal stem cells. *Tissue Eng Part A* 2015;**21**(19–20):2618–2628.

20 Yu B, Yang Y, Liu H, *et al.* Clusterin/Akt up-regulation is critical for GATA-4 mediated cytoprotection of mesenchymal stem cells against ischemia injury. *PLoS ONE* 2016;**11**(3):e0151542.

21 Kolesky DB, Homan KA, Skylar-Scott MA, Lewis JA. Three-dimensional bioprinting of thick vascularized tissues. *Proc Natl Acad Sci U S A* 2016;**113**(12):3179–3184.

22 Hao C, Yang S, Xu W, *et al.* MiR-708 promotes steroid-induced osteonecrosis of femoral head, suppresses osteogenic differentiation by targeting SMAD3. *Sci Rep* 2016;**6**:22599.

23 Yu J-L, Deng R, Chung SK, Chan GC-F. Epac activation regulates human mesenchymal stem cells migration and adhesion. *Stem Cells* 2016;**34**(4):948–959.

24 Consentius C, Akyüz L, Schmidt-Lucke JA, *et al.* Mesenchymal stromal cells prevent allostimulation in vivo and control checkpoints of Th1 priming: migration of human DC to lymph nodes and NK cell activation. *Stem Cells* 2015;**33**(10):3087–3099.

25 Holan V, Trosan P, Cejka C, *et al.* A comparative study of the therapeutic potential of mesenchymal stem cells and limbal epithelial stem cells for ocular surface reconstruction. *Stem Cells Transl Med* 2015;**4**(9):1052–1063.

26 Yue R, Zhou BO, Shimada IS, *et al.* Leptin receptor promotes adipogenesis and reduces osteogenesis by regulating mesenchymal stromal cells in adult bone marrow. *Cell Stem Cell* 2016; in press. doi: 10.1016/j.stem.2016.02.015.

27 Manzini BM, da Silva Santos Duarte A, Sankaramanivel S, *et al.* Useful properties of undifferentiated mesenchymal stromal cells and adipose tissue as the source in liver-regenerative therapy studied in an animal model of severe acute fulminant hepatitis. *Cytotherapy* 2015;**17**(8):1052–1065.

28 Lopez-Santalla M, Mancheño-Corvo P, Menta R, *et al.* Human adipose-derived mesenchymal stem cells modulate experimental autoimmune arthritis by modifying early adaptive T cell responses. *Stem Cells* 2015;**33**(12):3493–3503.

29 Kwon DY, Kwon JS, Park SH, *et al.* A computer-designed scaffold for bone regeneration within cranial defect using human dental pulp stem cells. *Sci Rep* 2015;**5**:12721.

30 Herberg S, Aguilar-Perez A, Howie RN, *et al.* Mesenchymal stem cell expression of SDF-1β synergizes with BMP-2 to augment cell-mediated healing of critical-sized mouse calvarial defects. *J Tissue Eng Regen Med* 2015; in press. doi: 10.1002/term.2078.

31 Winkler S, Hempel M, Brückner S, *et al.* Mouse white adipose tissue-derived mesenchymal stem cells gain pericentral and periportal hepatocyte features after differentiation *in vitro,* which are preserved *in vivo* after hepatic transplantation. *Acta Physiol (Oxf)* 2015;**215** (2):89–104.

32 Leyva-Leyva M, López-Díaz A, Barrera L, *et al.* Differential expression of adhesion-related proteins and MAPK pathways lead to suitable osteoblast differentiation of human mesenchymal stem cells subpopulations. *Stem Cells Dev* 2015;**24**(21):2577–2590.

33 Zhang Y, Jin Y, Lin Y, *et al.* Adipose-derived mesenchymal stem cells ameliorate ulcerative colitis through miR-1236 negatively regulating the expression of retinoid-related orphan receptor gamma. *DNA Cell Biol* 2015;**34**(10):618–625.

34 Ha CW, Park YB, Chung JY, Park YG. Cartilage repair using composites of human umbilical cord blood-derived mesenchymal stem cells and hyaluronic acid hydrogel in a minipig model. *Stem Cells Transl Med* 2015;**4**(9):1044–1051.

35 Correa D, Somoza RA, Lin P, *et al.* Mesenchymal stem cells regulate melanoma cancer cells extravasation to bone and liver at their perivascular niche. *Int J Cancer* 2016;**138**(2):417–427.

36 Xu G, Guo Y, Seng Z, *et al.* Bone marrow-derived mesenchymal stem cells co-expressing interleukin-18 and interferon-β exhibit potent antitumor effect against intracranial glioma in rats. *Oncol Rep* 2015;**34**(4):1915–1922.

37 Pollock K, Dahlenburg H, Nelson H, *et al.* Human mesenchymal stem cells genetically engineered to overexpress brain-derived neurotrophic factor improve outcomes in Huntington's disease mouse models. *Mol Ther* 2016; in press. doi: 10.1038/mt.2016.12.

38 Martino MM, Maruyama K, Kuhn GA, *et al.* Inhibition of IL-1R1/MyD88 signalling promotes mesenchymal stem cell-driven tissue regeneration. *Nat Commun* 2015;**7**:11051.

39 Kiernan J, Hu S, Grynpas MD, *et al.* Systemic mesenchymal stromal cell transplantation prevents functional bone loss in a mouse model of age-related osteoporosis. *Stem Cells Transl Med* 2016;**5**(5):683–693.

40 Mak J, Jablonski CL, Leonard CA, *et al.* Intra-articular injection of synovial mesenchymal stem cells improves cartilage repair in a mouse injury model. *Sci Rep* 2016;**6**:23076.

41 Assi R, Foster TR, He H, *et al.* Delivery of mesenchymal stem cells in biomimetic engineered scaffolds promotes healing of diabetic ulcers. *Regen Med* 2016;**11**(3):245–260.

42 Chan MC, Kuok DI, Leung CY, *et al.* Human mesenchymal stromal cells reduce influenza A H5N1-associated acute lung injury *in vitro* and *in vivo*. *Proc Natl Acad Sci U S A* 2016;**113**(13):3621–3626.

43 Kim JS, Cha SH, Kim WS, *et al.* A novel therapeutic approach using mesenchymal stem cells to protect against *Mycobacterium abscessus*. *Stem Cells* 2016; in press. doi: 10.1002/stem.2353.

44 Qiang Y, Liang G, Yu L. Human amniotic mesenchymal stem cells alleviate lung injury induced by ischemia and reperfusion after cardiopulmonary bypass in dogs. *Lab Invest* 2016; in press. doi: 10.1038/labinvest.2016.37.

45 Chiang ER, Ma HL, Wang JP, *et al.* Allogeneic mesenchymal stem cells in combination with hyaluronic acid for the treatment of osteoarthritis in rabbits. *PLoS ONE* 2016;**11**(2):e0149835.

46 Shi D, Zhang J, Zhou Q, *et al.* Quantitative evaluation of human bone mesenchymal stem cells rescuing fulminant hepatic failure in pigs. *Gut* 2016; in press. doi: 10.1136/gutjnl-2015-311146.

47 Mead B, Hill LJ, Blanch RJ, *et al.* Mesenchymal stromal cell-mediated neuroprotection and functional preservation of retinal ganglion cells in a rodent model of glaucoma. *Cytotherapy* 2016;**18**(4):487–496.

48 Maruyama T, Jeong J, Sheu T-J, Hsu W. Stem cells of the suture mesenchyme in craniofacial bone development, repair and regeneration. *Nat Commun* 2016;**7**:10526.

49 Zhou Z, You Z. Mesenchymal stem cells alleviate LPS-induced acute lung injury in mice by MiR-142a-5p-controlled pulmonary endothelial cell autophagy. *Cell Physiol Biochem* 2016;**38**(1): 258–266.

50 Wei JN, Cai F, Wang F, *et al.* Transplantation of CXCR4 overexpressed mesenchymal stem cells augments regeneration in degenerated intervertebral discs. *DNA Cell Biol* 2016; in press. doi: 10.1089/dna.2015.3118.

51 Kim DK, Nishida H, An SY, *et al.* Chromatographically isolated $CD63^{+}CD81^{+}$ extracellular vesicles from mesenchymal stromal cells rescue cognitive impairments after TBI. *Proc Natl Acad Sci U S A* 2016;**113**(1):170–175.

52 Perin EC, Borow KM, Silva GV, *et al.* A phase II dose-escalation study of allogeneic mesenchymal precursor cells in patients with ischemic or nonischemic heart failure. *Circ Res* 2015;**117**(6):576–584.

53 Petrou P, Gothelf Y, Argov Z, *et al.* Safety and clinical effects of mesenchymal stem cells secreting neurotrophic factor transplantation in patients with amyotrophic lateral sclerosis: results of phase 1/2 and 2a clinical trials. *JAMA Neurol* 2016;**73**(3):337–344.

54 Weiss DJ, Casaburi R, Flannery R, *et al.* A placebo-controlled, randomized trial of mesenchymal stem cells in COPD. *Chest* 2013;**143**(6):1590–1598.

55 Forbes GM, Sturm MJ, Leong RW, *et al.* A phase 2 study of allogeneic mesenchymal stromal cells for luminal Crohn's disease refractory to biologic therapy. *Clin Gastroenterol Hepatol* 2014;**12**(1):64–71.

CHAPTER 64

# Reconciling the stem cell and paracrine paradigms of mesenchymal stem cell function

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## 64.1 Summary

Nearly half a century has passed since Friedenstein and co-workers identified a stem cell population in bone marrow, distinct from the hematopoietic stem cell (HSC), with the capacity to generate a heterotopic bone marrow organ capable of supporting hematopoiesis. Ensuing decades of study progressively refined our knowledge about the physical nature of these cells, and their ability to generate skeletal tissue at the single cell level led to their characterization as mesenchymal stem cells (MSCs). In recent years the MSC concept has changed radically based on evidence that MSCs reside within the vasculature of most tissues and possess a broad range of biological activities that far exceed their skeletogenic potential. In the latter case MSCs have been shown to secrete a plethora of bioactive molecules as well as protein/RNA-laden microvesicles with angiogenic, anti-inflammatory, trophic, and immunomodulatory activities, which are believed to induce therapeutic effects *in vivo* via drug-like action. An important tenet of the revised MSC concept is that cells achieve therapeutic effects independent of their stem cell properties. Assimilating the vast amount of new data on MSC function with prior established principles has created much confusion and controversy in the field. As a result, there exists little consensus on how to operationally define the cells, and there is disagreement in the field as to whether MSC-based therapies exert beneficial effects of clinical relevance in the treatment of nonskeletal disorders in human patients. Herein, we provide a brief history of how the MSC perspective has evolved, discuss how major paradigm shifts have impacted the field of study, and propose a stem-cell-centric viewpoint of MSCs as a means to bring better continuity to the field.

## 64.2 Introduction

The field of research dedicated to the study of MSCs now spans five decades. During this time many theories have been put forth to explain the origin and biological functions of MSCs. However, these theories remain fragmented and incomplete. Efforts spearheaded by various societies and regulatory bodies to define the MSC at one time yielded a consensus opinion [1], but this definition is now regarded as woefully inadequate and no consensus on an updated definition has been achieved to date. This has prompted renewed efforts to redefine MSCs based on their potency in biological assays rather than their phenotypic characteristics and/or composition of matter. Achieving this goal, however, still requires a significant amount of consensus building, which may or may not be achievable. For example,

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choosing the most relevant biological potency assays to evaluate the overall quality of populations is a daunting task considering the breadth of reported activities ascribed to the cells (currently over 40 000 manuscripts), the limited knowledge regarding their mechanism of action *in vivo*, and the difficulty in translating outcomes from *in vitro* and preclinical animal studies to the human condition. This task is made even more difficult by the fact that one must develop a calibration standard with a range of activities in each potency assay against which experimental and clinical samples can be compared. However, no formalized standards exist for the isolation and manufacture of MSCs, and, as already noted, there is currently no consensus opinion on how to rigorously characterize the cells.

In retrospect, the controversy over how to define MSCs mirrors the evolving paradigms put forth to describe their biological function. MSCs were originally characterized as skeletal stem cells based on their capacity to generate heterotopic bone *in vivo*. However, the prevailing viewpoint now recognizes MSCs as a specialized stromal cell that modulates the immune system and promotes tissue regeneration independent of their stem cell properties [2–6]. As anticipated, the bulk of published research in recent years has focused on delineating these paracrine mechanisms, but many of these studies do not report on whether paracrine signaling also influences MSC growth, viability, and differentiation. We view this as a missed opportunity to integrate the two fields of thought, which view MSCs as stem cells or specialized stromal cells. Herein, we try to reconcile these two viewpoints by examining the stem cell and paracrine properties of MSCs in some detail, review evidence linking these two phenomena, and offer a framework rooted in stem cell biology to reconcile these opposing viewpoints in an effort to bring greater continuity to the field.

## 64.3 The stem cell paradigm revisited

In a series of seminal papers dating back to the 1960s, Friedenstein and co-workers were the first to describe a stem cell population in bone marrow that possessed intrinsic bone-forming ability. Two series of experiments in particular are most relevant to this discussion. The first demonstrated that the bone-forming cells in marrow were not of hematopoietic origin. To show this, Friedenstein transplanted bone marrow from a parental mouse strain under the renal capsule of an F1 hybrid and confirmed by karyotype analysis that the resulting ectopic bone originated from the parental strain and that hematopoietic cells that colonized the bone were derived from the F1 hybrid strain (Figure 64.1A) [7].

Friedenstein further demonstrated that, when the osseous tissue was transplanted back into the parental strain, bone tissue formation continued unimpeded but the colonizing hematopoietic cells were rejected. Friedenstein confirmed these findings by showing that male marrow cells recovered from a heterotopic ossicle and transplanted into syngeneic females immunized against male antigens failed to generate ectopic bone due to rejection of the transplanted cells by the host's immune system (Figure 64.1B) [8]. In the second set of experiments, Friedenstein demonstrated that the bone-forming cells in marrow were self-renewing based on their ability to regenerate new osseous tissue when transplanted successively into recipient mice [8]. Importantly, the self-renewing capacity of the cells was exhausted after passage through five recipients, which was similar to that reported for HSCs passed through irradiated recipients. Based on these findings, Friedenstein characterized the cells as osteogenic stem cells and went on to show that they resided in the plastic-adherent fraction of marrow and were capable of clonal expansion [9,10].

In the ensuing decades various laboratories developed *in vitro* assays to evaluate the differentiation potential of marrow-derived osteogenic stem cells [11–17], which culminated in studies confirming their ability to undergo trilineage differentiation at the level of a single cell [18,19]. In the latter case, analysis of a total of six single cell-derived clonal populations of human MSCs revealed that all clones underwent osteogenic differentiation, five underwent adipogenic differentiation, and two underwent

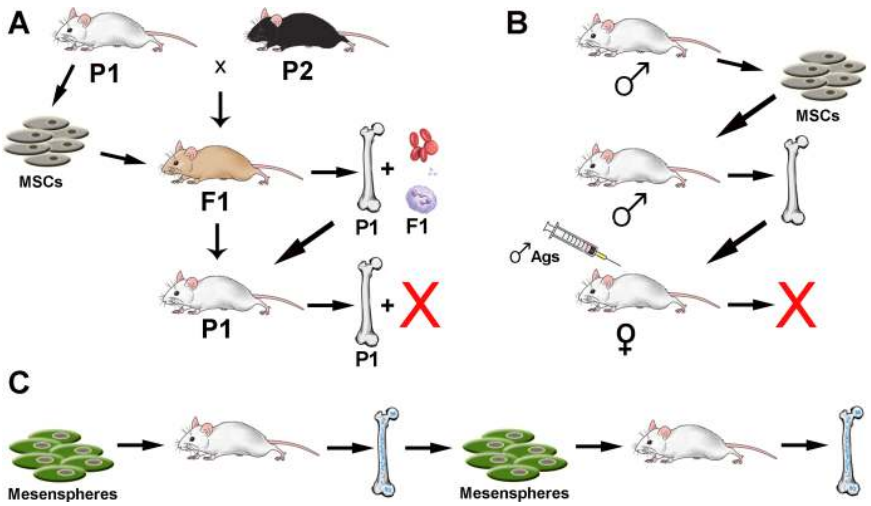



**Figure 64.1** MSCs are self-renewing stem cells. (A, B) Experimental paradigms used to demonstrate that skeletogenic stem cells in bone marrow (MSCs) are physically distinct from blood forming cells (HSCs). (C) Mesenspheres derived from triple transgenic *Nes*-GFP$^{+}$/Col2.3-cre/R26R mice serially regenerate a hematopoietic organ containing osteoblasts that express β-galactosidase when passed through multiple wild-type recipient mice. (See color plate section.)

chondrogenic differentiation [18]. The observation that not all colonies exhibited the full range of differentiation suggested that populations are an admixture of stem and progenitor cells of varying potency, which was subsequently confirmed by later studies (see later). Ironically, the significance of these findings was largely unheralded within the broader stem cell community. Nevertheless, more recent studies using genetic-based approaches have confirmed Friedenstein's original observations and further refined the physical identity and function of the MSC in marrow. Specifically, Méndez-Ferrer *et al.* [20] identified a population of stromal cells in bone marrow expressing the green fluorescent protein (GFP) under the regulatory elements of the Nestin promoter. These cells, referred to as *Nes*-GFP$^+$, exhibited a perivascular distribution in close proximity to CD150$^+$ CD48$^-$ Lin$^-$ HSCs within bone marrow but lacked expression of vascular endothelial cell markers such as CD31 and CD34 or vascular endothelial cadherin. Importantly, *Nes*-GFP$^+$ cells fulfilled the definition of an MSC since they comprised the entire colony-forming unit–fibroblast (an acknowledged marker of MSCs) fraction in marrow and differentiated into adipocytes, osteoblasts, and chondrocytes at the population level. Moreover, when clonally derived populations from triple transgenic *Nes*-GFP$^+$/Col2.3-cre/R26R mice (the *Nes-Gfp* line was intercrossed with a line expressing the Cre recombinase under the osteoblast-specific 2.3-kilobase proximal fragment of the α1(I)-collagen promoter and the *ROSA26/loxP-stop-loxP-lacZ* reporter line) were loaded into phosphocalcic ceramic ossicles and implanted subcutaneously into littermates that lacked the transgenes, the implanted cells generated heterotopic osseous tissue that contained β-galactosidase$^+$ osteoblasts associated with hematopoietic activity. Cells recovered from the ossicle regenerated GFP$^+$ mesenspheres *in vitro*, which when implanted into secondary recipients yielded osseous tissue containing Col2.3$^+$ osteoblasts that supported hematopoiesis (Figure 64.1C) [21]. Collectively, these studies demonstrate that *Nes*-GFP$^+$/PDGFRa$^+$ perivascular cells are capable of transferring and generating a bone marrow organ system *in vivo* and, as such, fulfill the definition of a skeletal stem cell.

## 64.4 The paracrine paradigm

Early efforts to exploit the stem cell properties of MSCs for a therapeutic intent were directed at treating children with osteogenesis imperfecta [22,23], an inherited skeletal disorder causing brittle bones, and to augment hematopoiesis in women with breast cancer undergoing HSC transplantation after high-dose chemotherapy [24]. Although these studies both yielded encouraging results, similar efforts were superseded by studies indicating that MSCs as well as other stem cell populations including HSCs and neural stem cells possessed unexpected plasticity, as evidenced by their transdifferentiation into cardiomyocytes, astrocytes, hepatocytes, lung epithelium, and other lineages (Figure 64.2) (reviewed by Wulf *et al.* [25] and Krause [26]).

These provocative results captivated the scientific community for nearly a decade, spurring the discovery of additional stem cell populations in bone marrow with broad plasticity, including multipotent adult progenitor cells [27], unrestricted somatic stem cells [28], and very small embryonic-like cells [29], but this was largely abandoned when mounting evidence suggested that the phenomenon occurred at a low frequency *in vivo* and therefore was not therapeutically meaningful [30,31].

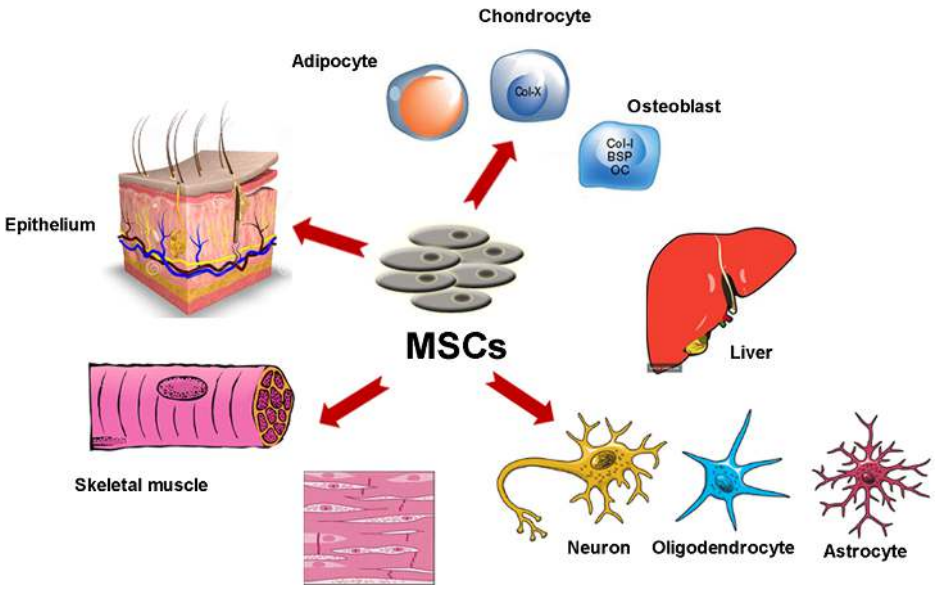



**Figure 64.2** MSC plasticity. MSCs are best characterized by their capacity to differentiate into connective tissue cell lineages but were also shown to adopt characteristics of epithelial cells, skeletal and smooth muscle, neural cell lineages, and hepatocytes (see text for references and current views). (See color plate section.)

An important consequence of this chapter in the MSC saga was that cells were administered to a myriad of animal species (mice, rats, sheep, swine, and nonhuman primates) affected by a broad range of injuries or diseases. Although evidence indicated that few MSCs survived when injected directly into sites of tissue injury [32], and that cells administered systemically embolized in the lungs and were rapidly cleared [33], short-term therapeutic benefits were consistently observed. For example, MSCs were found to reduce the overall infarct size and to increase the density of vascular beds in cardiac tissue subject to ischemia–reperfusion injury [34] (and see Chapter 45), ameliorate inflammation following acute lung injury [35,36], restore blood flow in chronic limb ischemia models [37] (and see Chapter 46), inhibit proliferation of mitogen-stimulated lymphocytes *in vitro* (and see Chapter 34), and prolong survival of autologous third-party donor skin grafts in baboons [38]. These and other findings led to a revision of the MSC concept (and a change in name) such that cells were believed to effect tissue repair by altering the tissue microenvironment via paracrine signaling rather than directed differentiation and cell replacement. This new paradigm gained support from genomics-based studies, which catalogued a large repertoire of expressed transcripts with angiogenic, anti-inflammatory, immunomodulatory, and trophic activity [39–41]. The concept was emboldened by subsequent studies demonstrating that MSC-conditioned media and more recently protein/RNA-laden microvesicles replicated some of the therapeutic effects seen following cell administration in animal models of disease [42–44] (see Chapter 38).

### 64.4.1 "Mesenchymal stem cell pharmacology": cells are not drug-like

The revisionist viewpoint that MSCs are specialized effector cells that promote tissue repair via paracrine action has gained significant traction, and ascribes to the notion that cells achieve therapeutic effects via drug-like action by a "hit and run" mechanism *in vivo* [45,46]. However, this viewpoint is not well substantiated by the published literature. For example, a fundamental principle of pharmacology is that a relationship exists between the concentration of a drug at its site of action and its beneficial or toxic effect. This link between pharmacokinetics and pharmacodynamics determines a drug's overall potency *in vivo*, establishes dose–response relationships, and predicts the effect–time course resulting from a drug dose. Consequently, if MSCs exhibit drug-like behavior then their clinically beneficial effects should depend both on dose and route of administration. However, few if any studies have demonstrated dose-dependent effects of MSCs in large animal models or human clinical trials, and in many cases route of administration does not appear to affect outcome. For example, in studies conducted in swine subjected to temporary balloon occlusion of the left descending coronary artery, one study failed to detect any significant effect on left ventricular ejection fraction (LVEF) or left ventricular volumes following endomyocardial delivery of 24 million to 440 million MSCs [47]. Another study demonstrated dose-independent effects on regional perfusion and left ventricular function following administration of 1 million to 10 million MSCs [48], while a third study reported a significant reduction in infarct size following administration of 200 million but not 20 million MSCs [49]. In related studies, Hamamoto *et al.* [50] reported that administration of 25 million or 75 million but not 225 million or 450 million stromal precursor antigen-$3^+$ MSCs significantly attenuated infarct expansion and increased left ventricular end diastolic and systolic volume in a sheep model of acute myocardial infarction. Finally, a meta-analysis of 52 preclinical studies employing bone marrow mononuclear cells or MSCs in a total of 888 large animals (swine, sheep, dog) subjected to myocardial infarction revealed a significant ($P < 0.01$) degree of heterogeneity and inconsistency between data sets but demonstrated a significant ($P < 0.001$) increase (7.5%) in LVEF after cell therapy versus control [51]. Multivariable meta-regression analysis revealed that cell type ($P = 0.04$) and type of infarction ($P = 0.045$) were the only independent significant predictors of LVEF improvement, and no trend in LVEF improvement was observed regarding animal model ($P = 0.5$) or route of cell delivery ($P = 0.9$).

As anticipated from the studies in large animals, MSC-based therapies have also yielded inconsistent results in human clinical trials. For example, a randomized controlled double-blind trial to assess the effect of allogeneic MSCs (trade name Prochymal™) in patients with acute myocardial infarction yielded the expected safety data, but notable differences in outcome measures did not exhibit dose responsiveness [52]. Similarly, the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis Pilot Study (POSEIDON) clinical trial of patients with ischemic cardiomyopathy demonstrated that MSCs yielded significant reductions in scar size with an inverse dose response: the effect of 20 million MSCs exceeded that of 200 million [53]. Additionally, a multicenter phase II study of 55 patients with corticosteroid-refractory acute graft-versus-host disease, a complication of allogeneic HSC transplantation, yielded a complete response in approximately half the patients administered MSCs and the 2-year probability of survival in complete responders was significantly better than that in patients with a partial or no response ($P = 0.018$) [54]. However, while clinically meaningful responses were obtained following infusion of as few as $0.8 \times 10^6$ MSCs/kg, doses as high as $1.9 \times 10^6$ MSCs/kg were not successful in all cases.

These studies demonstrate a lack of dose responsiveness of MSC-based therapies both in large animal models and human patients, thereby refuting the drug-like nature of the cells. Moreover, clinical trials have reported similar improvements in LVEF of patients with ischemic heart disease receiving intracoronary [53,55,56] or transendocardial MSC injections [52,53] (see Chapter 45). MSCs were also reported to yield similar therapeutic effects in a rat model of stroke following intracranial [57], intravenous [58], intracarotid [59], or intracerebral [60] infusion. Thus, the basis for the drug-like action of MSCs is not well substantiated by the literature. This is

expected based on fundamental differences between the processes governing the effects of drug administration (simple system) compared with a cell infusion (complex system) into a living organism (complex system).

### 64.4.2 Priming to enhance mesenchymal stem cell paracrine action also impacts cell growth and survival

The paracrine paradigm of MSC function postulates that cells respond to environmental cues by secreting bioactive factors that yield clinically beneficial effects. This concept explains why MSCs are efficacious across a broad spectrum of diseases even though bioactive factors they secrete may be contraindicated for a particular disease. For example, angiogenesis induced by tissue inflammation has been reported to worsen symptoms in rheumatoid arthritis, and vascular endothelial growth factor (VEGF, one of many proteins secreted by MSCs) is known to play a critical role in the pathogenic angiogenesis of rheumatoid arthritis [61]. Nevertheless, MSCs are being evaluated therapeutically in rheumatology owing to their ability to suppress inflammation and promote cartilage repair despite the fact that they are potently angiogenic [62]. Ironically, the literature is rife with studies that attempt to enhance the therapeutic potency of MSCs by culturing cells in various supplements (Table 64.1).

These studies inform that MSC potency is suboptimal in many experimental settings but also reveal insight into how these treatments alter MSC biology. For example, studies have shown that preconditioning regimens aimed at enhancing the angiogenic potential of MSCs also enhance cell proliferation and survival. Consistent with these studies, it is well established that FGF-2 and VEGF act synergistically to regulate angiogenesis during development [101,102], that secretion of these factors by MSCs is enhanced by hypoxic preconditioning [103], and that FGF-2 also promotes MSC growth, survival, and self-renewal [104–108] and delays the onset of cellular senescence during long-term culture expansion [109]. Other studies have shown that FGF-2 pretreatment enhances cellular differentiation, which seems counterintuitive, but most likely reflects its ability to preserve the progenitor pool during culture expansion. Thus, the angiogenic potential of MSCs appears to be linked to mechanisms controlling cell growth, survival, and self-renewal.

### 64.4.3 Licensing of immunomodulatory activity biases cell differentiation

In contrast, less is known about how conditions that enhance anti-inflammatory and immunomodulatory properties of MSCs affect their growth, survival, self-renewal, and differentiation. One agent that has been widely used is INF-γ. Krampera *et al.* initially demonstrated that the immunosuppressive activity of MSCs is induced in response to IFN-γ release from activated immune cells [110]. This "licensing" appears critical, since IFN-γ receptor-negative MSCs do not inhibit T cell functions and MSCs transplanted to IFN-γ knockout mice failed to ameliorate symptoms of GVHD [57]. When added as a media supplement, INF-γ has been reported to inhibit the growth of rodent and human MSCs [111], which is consistent with the known growth inhibitory effects of interferons (INFs) [112,113]. Moreover, Dugue *et al.* [114] showed that IFN-γ-inducible genes were upregulated during the early phases of osteoblast differentiation of human MSCs, which corresponded to a strong but transient increase in INF-γ production. Moreover, knockdown of INF-γ blocked osteogenic differentiation, and the addition of exogenous INF-γ accelerated the process. These effects of INF-γ were linked to changes in expression of *Runx2*. Furthermore, Vidal *et al.* [115] showed that the kynurenine pathway was activated in MSCs during commitment to the osteoblast lineage *in vitro* and that small-interfering-RNA-mediated knockdown or pharmacological inhibition of IDO strongly inhibited osteoblastogenesis. These data indicate that INF-γ-responsive pathways that confer immunosuppressive activity to MSCs also impact cellular differentiation.

MSCs have also been shown to express a subset of toll-like receptors (TLRs), and engagement of these receptors by their cognate ligands has been reported to modulate their anti-inflammatory and immunomodulatory activity [116]. Importantly, TLR activation does not appear to significantly impact the growth of MSCs isolated from different tissue sources [117–119] but does impact cellular differentiation (Table 64.2).

For example, studies have shown that TLR2 priming or TLR3/TLR4 priming inhibits adipogenic differentiation of adipose-derived [118] or bone-marrow-derived MSCs [65] respectively. In contrast, osteogenic differentiation was stimulated by TLR2/TLR3/TLR4 priming of adipose-derived MSCs [118,120], by TLR2/TLR4 priming of umbilical-cord-derived MSCs [119], and by TLR4 priming of bone-marrow-derived MSCs [65], whereas TLR9 [122] or TLR3 [65] priming inhibited osteogenic differentiation of the latter cells. Therefore, while differences in outcomes between these studies are muddled at best, they clearly indicate that TLR signaling also impacts cellular differentiation.

## 64.5 Modeling mesenchymal stem cell function using lessons learned from immunology

Efforts to model the immunomodulatory activity of MSCs have largely copied established paradigms in immunology. One model that has gained traction is the polarization of MSCs by TLR engagement to inflammatory (MSC 1) and anti-inflammatory (MSC 2) phenotypes (reviewed by Bernardo and Fibbe [116]). This is based on evidence showing that TLR3 ligation compared with TLR4 ligation induced distinct patterns of cytokine expression in MSCs [65]. However, close examination of this model reveals that the distinction between MSC 1 and MSC 2 phenotypes is not clearly demarcated based on the supporting data. For example, the pattern of cytokine expression in the two populations overlaps, and MSC 2 cells secrete chemokines such as CCL5 and CCL10 that are implicated in lymphocyte trafficking and bone remodeling rather than immunosuppression. Moreover,

**Table 64.1** Conditioning (pretreatment) regimens to enhance the paracrine functions of mesenchymal stromal cells.

| Pretreatment modification | Effect on mesenchymal stromal cells | Functional benefits | Reference |
|---|---|---|---|
| *Hematopoiesis* | | | |
| IL-1 | ↑ Growth<br>↑ Clonal efficiency | ↑ Hematopoietic support *in vitro* | [63] |
| *Anti-inflammatory and immunomodulatory activity* | | | |
| Substance P | ND | ↑ TGF-β secretion<br>↑ T cell suppressive activity of late-passage MSCs | [64] |
| TLR3 agonists | ND | ↑ Anti-inflammatory activity | [65] |
| TLR4 agonists | ND | ↑ Pro-inflammatory activity | [65,66] |
| ATP | ↓ Proliferation<br>↑ Migration | ↑ Bone marrow homing<br>↓ Inhibition of T cell proliferation | [67] |
| IFN-γ | ↑ IDO, B7H1 and B7DC expression | ↑ Inhibition of T cell proliferation<br>↑ Th1 cytokine secretion | [68] |
| TNF | ↑ TSG-6 secretion | ↓ TLR2/NFκB signaling in macrophages | [69] |
| 3D spheroids | ↑ TSG-6 secretion | ↑ Efficacy in peritonitis model | [70] |
| Serum from ARDS mice | ↑ IL-10 and IL-1RA secretion | ↑ Protection against endotoxemic lung injury | [71] |
| *Angiogenic and cardio-protective activity* | | | |
| Hypoxia | ↑ Survival<br>↑ Mobility | ↑ Efficacy in myocardial infarction<br>↑ Efficacy in hind limb ischemia | [72–74] |
| IP6K inhibitors | ↑ Survival | ↑ Antiapoptotic, proangiogenic, and antifibrotic properties in myocardial ischemia | [75] |
| Oxytocin | ND | ↑ Angiogenic/cardioprotective activity of diabetes-insulted MSCs | [76] |
| Melatonin | ↑ Survival | ↑ Pro-angiogenic activity<br>↑ Efficacy in ischemic kidney | [77] |
| Dinitrophenol | ↑ Angiogenins<br>↑ Cardiomyogenic factors | ND | [78] |
| Hypoxia/reoxygenation- | ND | ↑ Rescue of ischemic rat cortical neurons | [79] |
| Prolyl hydroxylase inhibitor | ↑ Survival | ↑ Angiogenesis *in vitro*<br>↑ Efficacy in ischemic heart | [80] |
| Pioglitazon | ↑ Cardiomyogenic differentiation | ↑ Cardiac function after acute myocardial infarction *in vivo* | [81] |
| HMOX1 overexpression | ↓ Oxidative stress<br>↓ Apoptosis<br>↑ VEGF secretion | ↑ Repair of ischemic myocardium | [82] |
| NT3 | ↑ VEGF, NGF, BDNF secretion | ↑ Angiogenesis in feet of diabetic mice | [83] |
| HIF-1α overexpression | ↑ Migration | ↑ Efficacy in rat myocardial infarction model | [84] |
| TLR4 knockout | ↑ Angiogenin secretion<br>↑ Proliferation | ↑ Cardioprotection in rat ischemia–reperfusion model | [85] |
| TGF-α | ↑ VEGF secretion | ↑ Functional recovery of rat ischemic heart | [86] |
| GSK3B overexpression | ↑ Survival<br>↑ VEGF secretion | ↑ Function of in ischemic heart | [87] |
| Hypoxia | ↑ Migration<br>↑ Homing | ↑ Repair of acute kidney injury | [88] |
| SDF-1 | ↑ Proliferation<br>↑ Survival<br>↑ Engraftment | ↑ Functional recovery of ischemic heart | [89] |
| BDNF overexpression | ND | ↑ Recovery in rat MCAO model | [90] |

(*continued*)

**Table 64.1** (*Continued*)

| Pretreatment modification | Effect on mesenchymal stromal cells | Functional benefits | Reference |
|---|---|---|---|
| *Neuroprotective activity* | | | |
| Sevoflurane | ↑ Survival<br>↑ Migration | ↑ Neuroprotective effects | [91] |
| Valproic acid | ↑ Migration<br>↑ Trophic factor secretion | ↑ Neuroprotective effects in spinal cord injury | [92,93] |
| *Tumor interaction* | | | |
| Hypoxia | ↑ TGF-β1 secretion | ↑ Breast cancer progression | [94] |
| TNF | ↑ TRIAL, DKK3, TRL3 expression | ↑ Tumor suppressive activity | [95] |
| Astragaloside IV | ↑ Proliferation<br>↓ TLR4 | ND | [96] |
| 17β-Estradiol | ↓ $H_2O_2$-induced apoptosis | ND | [97] |
| Barberin | ↓ Hypoxia-induced apoptosis | ND | [98] |
| G-CSF | ↑ Viability | | [99] |
| FGF-2 | ↑ Liver genes | ↑ Homing to liver<br>↓ Liver fibrosis | [100] |

IL: interleukin; TGF: transforming growth factor; TLR: toll-like receptor; ATP: adenosine-5′-triphosphate; IFN: interferon; TNF: tumor necrosis factor; IDO: indoleamine 2,3-dioxygenase; B7H1: B7 homolog (also known as PD-L1 and CD274); B7DC: B7 dendritic cell (also known as PD-L2, CD273); Th1: type 1T helper cell; TSG-6: TNF-α-induced protein-6; NFκB: nuclear factor kappa light chain-enhancer of activated B cells; IL-1RA: IL-1 receptor antagonist; 3D: three-dimensional; ARDS: acute respiratory distress syndrome; IP6K: inositol hexakisphosphate kinase; HMOX1: heme oxygenase 1; NGF: nerve growth factor; BDNF: brain-derived neurotrophic factor; HIF-1α: hypoxia-inducible factor-1α; GSK: glycogen synthase kinase; SDF-1: stromal cell-derived factor-1 (also known as C–X–C motif chemokine ligand 12 (CXCL12)); MCAO: middle cerebral artery occlusion; TRAIL: TNF-related apoptosis-inducing ligand (also known as CD253 and TNF (ligand) superfamily, member 10 (TNFSF10)); DKK: dikkopf factor; $H_2O_2$: hydrogen peroxide; G-CSF: granulocyte colony-stimulating factor; FGF: fibroblast growth factor; ND: not determined.

**Table 64.2** Effect of TLRligands on growth and trilineage differentiation of MSCs.

| Cell type | Ligand/TLR | Growth | Adipo | Osteo | Chondro | Reference |
|---|---|---|---|---|---|---|
| ADMSCs | PGN/TLR2<br>LPS/TLR4<br>CpG/TLR9 | –<br>–<br>↓ | ↓<br>— | ↑<br>↑– | –<br>— | [118] |
| BM-MSCs | LPS/TLR4<br>Poly(I:C)/TLR3 | — | — | — | — | [117] |
| ADMSCs | Poly(I:C)/TLR3<br>LPS/TLR4 | — | — | ↑<br>↑ | ND<br>ND | [120] |
| UCMSCs | Pam(3)/TLR2<br>LPS/TLR4 | — | — | ↑<br>↑ | ↑<br>↑ | [119] |
| BM-MSCs | PGN/TLR2<br>Poly(I:C)/TLR3<br>LPS/TLR4 | ND<br>ND<br>ND | ND<br>ND<br>ND | –<br>— | ND<br>ND<br>ND | [121] |
| BM-MSCs | CpG/TLR9 | ↓ | ND | ↓ | ND | [122] |
| BM-MSCs | Poly(I:C)/TLR3<br>LPS/TLR4 | ND<br>ND | ↓<br>↓ | ↓<br>↑ | ↓– | [65] |

Adipo: adipogenesis; Osteo: osteogenesis; Chondro: chondrogenesis; ADMSCs: adipose-tissue-derived mesenchymal stromal cells; BM-MSCs: bone-marrow-derived mesenchymal stromal cells; UCMSCs: umbilical-cord-derived mesenchymal stromal cells; LPS: lipopolysaccharide; PGN: peptidoglycan; CpG: cytosine–phosphate–guanosine; polyI:C: polyinosinic:polycytidylic acid; Pam(3): Pam3CSK4 is a synthetic triacylated lipopeptide (LP) that mimics the acylated amino terminus of bacterial LPs; ND: not determined.

MSC 1 cells were characterized by high IL-6 expression, a characteristic of nonmanipulated MSCs, and both populations failed to inhibit T cell proliferation in a mixed lymphocyte reaction *in vitro*. Finally, the fact that TLR3-primed cells (MSC 2) expressed high levels of IDO mRNA and prostaglandin $E_2$ protein indicates that some overlap exists in the cellular response to TLR agonists and INF-γ.

Studies assessing how TLR engagement alters MSC function have also yielded inconsistent results. For example, several studies have reported that TLR engagement suppressed the ability of MSCs to inhibit T cell proliferation in response to allogeneic stimulation [117,121] and reduced MSC-mediated expansion of $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T cells [123], while others have reported no effects on immunomodulatory activity [124]. Moreover, in one study, TLR ligation did not affect prostaglandin $E_2$ or IDO levels but rather altered Jagged 1 levels [117]. In another study, TLR3/TLR4 priming enhanced IDO expression due to autocrine INF-β signaling [125]. Consequently, it is difficult to imagine how these complex and in some cases contradictory results can be distilled down to a simple unifying model of cell function.

## 64.6 A stem-cell-centric view of mesenchymal stem cells

Significant strides have been made in cataloging the nature and activity of paracrine factors secreted by MSCs and advancing MSC-based therapies for the treatment of ischemic and immune-mediated disorders in humans. Accordingly, much of the field has abandoned the stem-cell-centric view of MSCs, which has impeded efforts to model their functional complexity using established principles of stem cell biology. We posit that a stem-cell-centric framework may adequately encompass the paracrine functions ascribed to MSCs, and in doing so bring better continuity to the field. In this framework, cell fate changes manifest as changes in cell potency, both from perspectives of paracrine action and differentiation potential. This framework has similarity to that of the hematopoietic system and parallels that recently described for T helper cell identity [126,127].

With regard to MSCs, we propose that a naive MSC is a $Nestin^+$/$PDFRA^+$ perivascular cell that expresses high levels of angiogenins and hematopoietic growth factors, including IL-6, CXCL12, chemokine C–C motif ligand 4, and stem cell factor. Importantly, this cell undergoes self-renewal and possesses the capacity to transfer and generate the hematopoietic microenvironment *in vivo*. The proangiogenic nature of the naive MSC is best illustrated by studies showing that their capacity to generate heterotopic osseous tissue is dependent, in part, on secretion of angiopoietin 1 [128]. Moreover, cytokines produced by MSCs that regulate homing and retention of HSCs in bone marrow, such as stem cell factor [129] and SDF-1 (CXCL12) [130], also exhibit angiogenic activity, while angiogenins including VEGF [131] and angiopoietin 1 [132] play important roles in HSC maintenance. Furthermore, the fact that MSCs exist in a low-oxygen environment *in vivo* explains why hypoxia preconditioning enhances their growth, surviva,l and proangiogenic activity *in vitro* (Table 64.1). Importantly, naive MSCs also secrete chemokines that modulate lymphocyte trafficking, and therefore may promote retention of leukocytes within injured tissues. However, we are unaware of any studies indicating that administration of naive MSCs induces a robust inflammatory response, which is consistent with the fact that these cells secrete little if any IL-1α, IL-1β, or TNF. Indeed, a recent study indicated that MSCs do not produce TNF even after LPS stimulation due to the fact that the TNF promoter is heavily methylated [133]. Based on these data, we argue that naive MSCs are not proinflammatory *per se*, but rather are not committed to an anti-inflammatory phenotype.

Consistent with this framework, few, if any, studies have reported that preconditioning regimens that enhance anti-inflammatory or immunomodulatory activity also promote MSC proliferation or enhance the angiogenic potential of MSCs. In contrast, one recent study showed that MSCs cultured in osteogenic induction medium secreted significantly lower levels of VEGF and several other angiogenins compared with cells cultured in growth media, and they were also less potent in supporting proliferation, migration, and tubule formation of human umbilical cord blood endothelial cells [134]. VEGF has been reported to inhibit adipocyte differentiation by regulating Runx2 and peroxisome proliferator-activated receptor-γ (PPAR-γ) levels in MSCs and reduced levels of VEGF during aging coincides with loss of trabecular bone mass and an increase in marrow fat [135,136]. These data suggest that the stem cell functions of MSCs are reinforced at the expense of effector functions and that acquisition of the latter occurs via a hierarchical process similar to that described for trilineage differentiation (Figure 64.3).

Indeed, clonal-based studies have definitively shown that MSC populations contain an admixture of all eight possible categories of trilineage progenitors [137–140] and that clones of varying potency exhibit significant differences in growth rates, colony-forming potential, and apoptosis [141]. These data reinforce the notion that cell differentiation is deterministic and that commitment to one lineage occurs at the expense of other possible fates.

Based on this paradigm, we argue that effector functions of MSCs are regulated by a similar process and that adoption of an effector cell fate represents a continuum within the hierarchy of cell specification for the naive MSCs (Figure 64.4).

This framework is consistent with the fact that INF-γ priming, which induces potent immunomodulatory activity, also impairs MSC growth and biases their differentiation toward a given connective tissue lineage. Bias is possible because commitment of naive cells to an "effector" phenotype induces expression of the master regulators *RUNX2*, *PPAR-γ*, and *SOX9*, which drive commitment to osteogenic, adipogenic, and chondrogenic fates respectively. Importantly, TLR activation has also been shown to alter the capacity of bone-marrow-derived MSCs to support

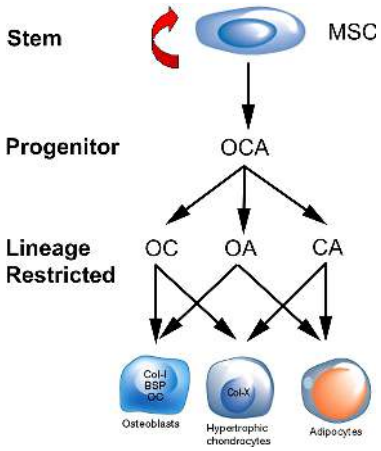



**Figure 64.3** Trilineage differentiation of MSCs follows a hierarchical process. The schematic illustrates the commitment of an MSC to a trilineage progenitor, the potency of which becomes progressively more restricted as differentiation proceeds down the hierarchy. (See color plate section.)

$CD34^+$ cells in an undifferentiated state [142] and MSCs differentiated to adipocytes no longer suppress, but enhance, the growth of alloantigen- or mitogen-stimulated T cells [143]. Similar to the process described for T helper cells, changes in MSC fate are nuanced, in that effector cells retain plasticity in their ability to respond to environmental cues. This plasticity

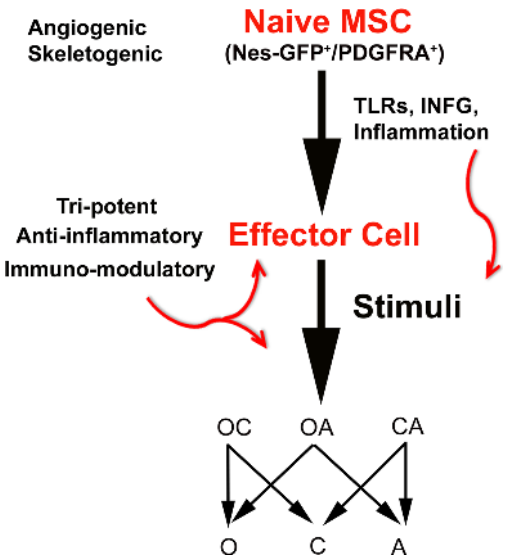



**Figure 64.4** Stem-cell-centric framework of MSC function. The schematic illustrates the naive MSC as an angiogenic perivascular cell capable of generating a hematopoietic organ. The naïve MSC is induced to adopt an effector phenotype by deterministic (specific stimuli) or stochastic processes, which induce expression of paracrine factors with anti-inflammatory and immunomodulatory activity. This fate change also derepresses pathways that drive cell differentiation toward connective tissue lineages, thereby allowing external stimuli to bias commitment toward these lineages. Overt cellular differentiation also alters the paracrine function of cells by suppressing their anti-inflammatory and immunomodulatory activities. (See color plate section.)

may be regulated by multiple mechanisms. For example, cells may retain the ability to rapidly secrete cytokines and chemokines in response to external stimuli by epigenetic regulation of gene transcription and posttranslational modification of protein function and stability. Moreover, cell fate changes may be reinforced by antagonistic or competitive processes; for example, those that drive self-renewal impede cell differentiation and/or feedback inhibition of entire signaling pathways. An important aspect of this framework is that it provides a working model that can be readily tested. As noted above, evidence exists that changes in effector function influence the growth, survival, hematopoiesis-supporting activity, and differentiation of MSCs. Therefore, if these pathways are mechanistically linked, as suggested by these data, it should be straightforward to evaluate in a rigorous manner how INF-γ priming, as an example, alters the ability of MSCs to serially regenerate heterotopic osseous tissue *in vivo* or provide a therapeutic benefit in experimental models of ischemic injury.

## 64.7 Closing remarks

The relative ease by which MSCs are procured and expanded *in vitro*, together with their lack of infusion-related toxicity in humans, has spurred their use for treating a large number of human diseases. However, MSC-based therapies to date have yielded inconsistent and suboptimal clinical benefits. We believe that the paradigm-centric viewpoint of the MSC has been oversimplified and overemphasized. As a result, much of the research devoted to this topic has been conducted in a manner that ignores other basic aspects of MSC biology. Nevertheless, as outlined in this chapter, we believe ample evidence exists to suggest that stem cell and effector functions of MSCs are mechanistically linked, and it is this link that serves as the basis for the stem-cell-centric framework proposed here. Our hope is that this provocative hypothesis will encourage future studies to employ a systems biology approach and examine how preconditioning or licensing of MSC paracrine function impacts not only on their therapeutic potency but also on their stem cell properties.

## References

1 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent esenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**:315–317.
2 D. J. Prockop. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;**276**:71–74.
3 A. I. Caplan. The mesengenic process. *Clin Plast Surg* 1994;**21**:429–435.
4 A. I. Caplan and J. E. Dennis. Mesenchymal stem cells as trophic mediators. *J Cell Biochem* 2006;**98**:1076–1084.
5 J. R. Lavoie and M. Rosu-Myles. Uncovering the secretes of mesenchymal stem cells. *Biochimie* 2013;**95**:2212–2221.

6 J. Tolar, K. Le Blanc, A. Keating, and B. R. Blazar. Concise review: hitting the right spot with mesenchymal stromal cells. *Stem Cells* 2010;**28**:1446–1455.

7 A. J. Friedenstein, K. V. Petrakova, A. I. Kurolesova, and G. P. Frolova. Heterotopic of bone marrow. Analysis of precursor cells for osteogenic and hematopoietic tissues. *Transplantation* 1968;**6**:230–247.

8 A. Friedenstein and A. I. Kuralesova. Osteogenic precursor cells of bone marrow in radiation chimeras. *Transplantation* 1971;**12**:99–108.

9 A. J. Friedenstein, R. K. Chailakhjan, and K. S. Lalykina. The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. *Cell Tissue Kinet* 1970;**3**:393–403.

10 A. J. Friedenstein, U. F. Deriglasova, N. N. Kulagina, *et al.* Precursors for fibroblasts in different populations of hematopoietic cells as detected by the *in vitro* colony assay method. *Exp Hematol* 1974;**2**:83–92.

11 P. S. Leboy, J. N. Beresford, C. Devlin, and M. E. Owen. Dexamethasone induction of osteoblast mRNAs in rat marrow stromal cell cultures. *J Cell Physiol* 1991;**146**:370–378.

12 J. N. Beresford, C. J. Joyner, C. Devlin, and J. T. Triffitt. The effects of dexamethasone and 1,25-dihydroxyvitamin D3 on osteogenic differentiation of human marrow stromal cells in vitro. *Arch Oral Biol* 1994;**39**:941–947.

13 C. Maniatopoulos, J. Sodek, and A. H. Melcher. Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats. *Cell Tissue Res* 1988;**254**:317–330.

14 J. M. Gimble, M. A. Dorheim, Q. Cheng, *et al.* Response of bone marrow stromal cells to adipogenic antagonists. *Mol Cell Biol* 1989;**9**:4587–4595.

15 J. N. Beresford, J. H. Bennett, C. Devlin, *et al.* Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. *J Cell Sci* 1992;**102**(Pt 2):341–351.

16 B. Johnstone, T. M. Hering, A. I. Caplan, *et al. In vitro* chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res* 1998;**238**:265–272.

17 A. M. Mackay, S. C. Beck, J. M. Murphy, *et al.* Chondrogenic differentiation of cultured human mesenchymal stem cells from marrow. *Tissue Eng* 1998;**4**:415–428.

18 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**:143–147.

19 J. E. Dennis, A. Merriam, A. Awadallah, *et al.* A quadripotential mesenchymal progenitor cell isolated from the marrow of an adult mouse. *J Bone Miner Res* 1999;**14**:700–709.

20 S. Méndez-Ferrer, T. V. Michurina, F. Ferraro, *et al.* Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. *Nature* 2010;**466**:829–834.

21 J. Isern, A. García-García, A. M. Martín, *et al.* The neural crest is a source of mesenchymal stem cells with specialized hematopoietic stem cell niche function. *Elife* 2014;**3**:e03696.

22 E. M. Horwitz, D. J. Prockop, L. A. Fitzpatrick, *et al.* Transplantability and therapeutic effects of bone marrow-derived mesenchymal cells in children with osteogenesis imperfecta. *Nat Med* 1999;**5**:309–313.

23 E. M. Horwitz, P. L. Gordon, W. K. Koo, *et al.* Isolated allogeneic bone marrow-derived mesenchymal cells engraft and stimulate growth in children with osteogenesis imperfecta: Implications for cell therapy of bone. *Proc Natl Acad Sci U S A* 2002;**99**:8932–8937.

24 O. N. Koç, S. L. Gerson, B. W. Cooper, *et al.* Rapid hematopoietic recovery after coinfusion of autologous-blood stem cells and culture-expanded marrow mesenchymal stem cells in advanced breast cancer patients receiving high-dose chemotherapy. *J Clin Oncol* 2000;**18**:307–316.

25 G. G. Wulf, K. A. Jackson, and M. A. Goodell. Somatic stem cell plasticity: current evidence and emerging concepts. *Exp Hematol* 2001;**29**:1361–1370.

26 D. S. Krause. Plasticity of marrow-derived stem cells. *Gene Ther* 2002;**9**:754–758.

27 Y. Jiang, B. N. Jahagirdar, R. L. Reinhardt, *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002;**418**:41–49 (erratum in *Nature* 2007; **447**(7146): 879–880)).

28 G. Kogler, S. Sensken, J. A. Airey, *et al.* A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *J Exp Med* 2004;**200**:123–135.

29 M. Kucia, R. Reca, F. R. Campbell, *et al.* A population of very small embryonic-like (VSEL) $CXCR4^+SSEA\text{-}1^+Oct\text{-}4^+$ stem cells identified in adult bone marrow. *Leukemia* 2006;**20**:857–869.

30 M. A. Goodell. Stem-cell "plasticity": befuddled by the muddle. *Curr Opin Hematol* 2003;**10**:208–213.

31 M. Raff. Adult stem cell plasticity: fact or artifact? *Annu Rev Cell Dev Biol* 2003;**19**:1–22.

32 C. Toma, W. R. Wagner, S. Bowry, *et al.* Fate of culture-expanded mesenchymal stem cells in the microvasculature: *in vivo* observations of cell kinetics. *Circ Res* 2009;**104**:398–402.

33 R. H. Lee, A. A. Pulin, M. J. Seo, *et al.* Intravenous hMSCs improve myocardial infarction in mice because cells embolized in lung are activated to secrete the anti-inflammatory protein TSG-6. *Cell Stem Cell* 2009;**5**:54–63.

34 J. G. Shake, P. J. Gruber, W. A. Baumgartner, *et al.* Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. *Ann Thorac Surg* 2002;**73**:1919–1925, discussion 1926.

35 L. A. Ortiz, F. Gambelli, C. McBride, *et al.* Mesenchymal stem cell engraftment in lung is enhanced in response to bleomycin exposure and ameliorates its fibrotic effects. *Proc Natl Acad Sci U S A* 2003;**100**:8407–8411.

36 M. Rojas, J. Xu, C. R. Woods, *et al.* Bone marrow-derived mesenchymal stem cells in repair of the injured lung. *Am J Respir Cell Mol Biol* 2005;**33**:145–152.

37 A. Al-Khaldi, H. Al-Sabti, J. Galipeau, and K. Lachappelle. Therapeutic angiogenesis using autologous bone marrow stromal cells: improved blood flow in a chronic limb ischemia model. *Ann Thorac Surg* 2003;**75**:204–209.

38 A. Bartholomew, C. Sturgeon, M. Siatskas, *et al.* Mesenchymal stem cells suppress lymphocyte proliferation *in vitro* and prolong skin graft survival *in vivo*. *Exp Hematol* 2002;**30**:42–48.

39 N. Tremain, J. Korkko, D. Ibberson, *et al.* MicroSAGE analysis of 2,353 expressed genes in a single cell-derived colony of undifferentiated human mesenchymal stem cells reveals mRNAs of multiple cell lineages. *Stem Cells* 2001;**19**:408–418.

40 W. A. Silva Jr, D. T. Covas, R. A. Panepucci, *et al.* The profile of gene expression of human marrow mesenchymal stem cells. *Stem Cells* 2003;**21**:661–669.

41 D. G. Phinney, K. Hill, C. Michelson, *et al.* Biological activities encoded by the murine mesenchymal stem cell transcriptome provide a basis for their developmental potential and broad therapeutic efficacy. *Stem Cells* 2006;**24**:186–198.

42 L. Timmers, S. K. Lim, F. Arslan, *et al.* Reduction of myocardial infarct size by human mesenchymal stem cell conditioned medium. *Stem Cell Res* 2007;**1**:129–137.

43 S. C. Hung, R. R. Pochampally, S. C. Chen, *et al.* Angiogenic effects of human multipotent stromal cell conditioned medium activate the PI3K–Akt pathway in hypoxic endothelial cells to inhibit apoptosis, increase survival, and stimulate angiogenesis. *Stem Cells* 2007;**25**:2363–2370.

44 R. C. Lai, F. Arslan, M. M. Lee, *et al.* Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. *Stem Cell Res* 2010;**4**:214–222.

45 L. von Bahr, I. Batsis, G. Moll, *et al.* Analysis of tissues following mesenchymal stromal cell therapy in humans indicates limited long-term engraftment and no ectopic tissue formation. *Stem Cells* 2012;**30**:1575–1578.

46 J. A. Ankrum, J. F. Ong, and J. M. Karp. Mesenchymal stem cells: immune evasive, not immune privileged. *Nat Biotechnol* 2014;**32**:252–260.

47 S. M. Hashemi, S. Ghods, F. D. Kolodgie, *et al.* A placebo controlled, dose-ranging, safety study of allogenic mesenchymal stem cells injected by endomyocardial delivery after an acute myocardial infarction. *Eur Heart J* 2008;**29**:251–259.

48 M. E. Halkos, Z. Q. Zhao, F. Kerendi, *et al.* Intravenous infusion of mesenchymal stem cells enhances regional perfusion and improves ventricular function in a porcine model of myocardial infarction. *Basic Res Cardiol* 2008;**103**:525–536.

49 K. H. Schuleri, G. S. Feigenbaum, M. Centola, *et al.* Autologous mesenchymal stem cells produce reverse remodelling in chronic ischaemic cardiomyopathy. *Eur Heart J* 2009;**30**:2722–2732.

50 H. Hamamoto, J. H. Gorman III L. P. Ryan, *et al.* Allogeneic mesenchymal precursor cell therapy to limit remodeling after myocardial infarction: the effect of cell dosage. *Ann Thorac Surg* 2009;**87**:794–801.

51 T. I. van der Spoel, S. J. Jansen of Lorkeers, P. Agostoni, *et al.* Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. *Cardiovasc Res* 2011;**91**:649–658.

52 J. M. Hare, J. H. Traverse, T. D. Henry, *et al.* A randomized, double-blind, placebo-controlled, dose-escalation study of intravenous adult human mesenchymal stem cells (Prochymal) after acute myocardial infarction. *J Am Coll Cardiol* 2009;**54**:2277–2286.

53 J. M. Hare, J. E. Fishman, G. Gerstenblith, *et al.* Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA* 2012;**308**:2369–2379.

54 K. Le Blanc, F. Frassoni, L. Ball, *et al.* Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. *Lancet* 2008;**371**:1579–1586.

55 S. Chen, Z. Liu, N. Tian, *et al.* Intracoronary transplantation of autologous bone marrow mesenchymal stem cells for ischemic cardiomyopathy due to isolated chronic occluded left anterior descending artery. *J Invasive Cardiol* 2006;**18**:552–556.

56 J. W. Lee, S. H. Lee, Y. J. Youn, *et al.* A randomized, open-label, multicenter trial for the safety and efficacy of adult mesenchymal stem cells after acute myocardial infarction. *J Korean Med Sci* 2014;**29**:23–31.

57 A. Mahmood, D. Lu, L. Yi, *et al.* Intracranial bone marrow transplantation after traumatic brain injury improving functional outcome in adult rats. *J Neurosurg* 2001;**94**:589–595.

58 J. Chen, Y. Li, L. Wang, *et al.* Therapeutic benefit of intravenous administration of bone marrow stromal cells after cerebral ischemia in rats. *Stroke* 2001;**32**:1005–1011.

59 Y. Li, J. Chen, L. Wang, *et al.* Treatment of stroke in rat with intracarotid administration of marrow stromal cells. *Neurology* 2001;**56**:1666–1672.

60 J. Chen, Y. Li, L. Wang, *et al.* Therapeutic benefit of intracerebral transplantation of bone marrow stromal cells after cerebral ischemia in rats. *J Neurol Sci* 2001;**189**:49–57.

61 C. H. Wang, H. Yao, L. N. Chen, *et al.* CD147 induces angiogenesis through a vascular endothelial growth factor and hypoxia-inducible transcription factor 1α-mediated pathway in rheumatoid arthritis. *Arthritis Rheum* 2012;**64**:1818–1827.

62 M. Maumus, D. Guérit, K. Toupet, *et al.* Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. *Stem Cell Res Ther* 2011;**2**:14.

63 A. E. Bigildeev, E. A. Zezina, I. N. Shipounova, and N. J. Drize. Interleukin-1β enhances human multipotent mesenchymal stromal cell proliferative potential and their ability to maintain hematopoietic precursor cells. *Cytokine* 2014;**71**:246–254.

64 Y. Jin, H. S. Hong, and Y. Son. Substance P enhances mesenchymal stem cells-mediated immune modulation. *Cytokine* 2014;**71**:145–153.

65 R. S. Waterman, S. L. Tomchuck, S. L. Henkle, and A. M. Betancourt. A new mesenchymal stem cell (MSC) paradigm: polarization into a pro-inflammatory MSC1 or an immunosuppressive MSC2 phenotype. *PLoS ONE* 2010;**5**:e10088.

66 R. Romieu-Mourez, M. François, M. N. Boivin, *et al.* Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. *J Immunol* 2009;**182**:7963–7973.

67 D. Ferrari, S. Gulinelli, V. Salvestrini, *et al.* Purinergic stimulation of human mesenchymal stem cells potentiates their chemotactic response to CXCL12 and increases the homing capacity and production of proinflammatory cytokines. *Exp Hematol* 2011;**39**:360–374, 374.e1–5.

68 R. Chinnadurai, I. B. Copland, S. R. Patel, and J. Galipeau. IDO-independent suppression of T cell effector function by IFN-gamma-licensed human mesenchymal stromal cells. *J Immunol* 2014;**192**:1491–1501.

69 H. Choi, R. H. Lee, N. Bazhanov, *et al.* Anti-inflammatory protein TSG-6 secreted by activated MSCs attenuates zymosan-induced mouse peritonitis by decreasing TLR2/NF-κB signaling in resident macrophages. *Blood* 2011;**118**:330–338.

70 T. J. Bartosh, J. H. Ylöstalo, A. Mohammadipoor, *et al.* Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. *Proc Natl Acad Sci U S A* 2010;**107**:13724–13729.

71 M. L. Bustos, L. Huleihel, E. M. Meyer, *et al.* Activation of human mesenchymal stem cells impacts their therapeutic abilities in lung injury by increasing interleukin (IL)-10 and IL-1RN levels. *Stem Cells Transl Med* 2013;**2**:884–895.

72 I. Rosova, M. Dao, B. Capoccia, *et al.* Hypoxic preconditioning results in increased motility and improved therapeutic potential

of human mesenchymal stem cells. *Stem Cells* 2008;**26**: 2173–2182.

73 X. Hu, R. Wu, Z. Jiang, *et al*. Leptin signaling is required for augmented therapeutic properties of mesenchymal stem cells conferred by hypoxia preconditioning. *Stem Cells* 2014;**32**:2702–2713.

74 Y. L. Tang, W. Zhu, M. Cheng, *et al*. Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression. *Circ Res* 2009;**104**:1209–1216.

75 Z. Zhang, D. Liang, X. Gao, *et al*. Selective inhibition of inositol hexakisphosphate kinases (IP6Ks) enhances mesenchymal stem cell engraftment and improves therapeutic efficacy for myocardial infarction. *Basic Res Cardiol* 2014;**109**:417.

76 Y. S. Kim, J. S. Kwon, M. H. Hong, *et al*. Restoration of angiogenic capacity of diabetes-insulted mesenchymal stem cells by oxytocin. *BMC Cell Biol* 2013;**14**:38.

77 C. Mias, E. Trouche, M. H. Seguelas, *et al. Ex vivo* pretreatment with melatonin improves survival, proangiogenic/mitogenic activity, and efficiency of mesenchymal stem cells injected into ischemic kidney. *Stem Cells* 2008;**26**:1749–1757.

78 A. Ali, M. A. Akhter, K. Haneef, *et al*. Dinitrophenol modulates gene expression levels of angiogenic, cell survival and cardiomyogenic factors in bone marrow derived mesenchymal stem cells. *Gene* 2015;**555**:448–457.

79 Y. S. Kim, M. Y. Noh, K. A. Cho, *et al*. Hypoxia/reoxygenation-preconditioned human bone marrow-derived mesenchymal stromal cells rescue ischemic rat cortical neurons by enhancing trophic factor release. *Mol Neurobiol* 2015;**52**:792–803.

80 X. Liu, J. A. Wang, X. Y. Ji, *et al*. Preconditioning of bone marrow mesenchymal stem cells by prolyl hydroxylase inhibition enhances cell survival and angiogenesis *in vitro* and after transplantation into the ischemic heart of rats. *Stem Cell Res Ther* 2014;**5**:111.

81 D. Shinmura, I. Togashi, S. Miyoshi, *et al*. Pretreatment of human mesenchymal stem cells with pioglitazone improved efficiency of cardiomyogenic transdifferentiation and cardiac function. *Stem Cells* 2011;**29**:357–366.

82 T. Tsubokawa, K. Yagi, C. Nakanishi, *et al*. Impact of anti-apoptotic and anti-oxidative effects of bone marrow mesenchymal stem cells with transient overexpression of heme oxygenase-1 on myocardial ischemia. *Am J Physiol Heart Circ Physiol* 20101;**298**: H1320–H1329 (erratum in *Am J Physiol Heart Circ Physiol* 2012; **303**(4): H496).

83 L. Shen, W. Zeng, Y. X. Wu, *et al*. Neurotrophin-3 accelerates wound healing in diabetic mice by promoting a paracrine response in mesenchymal stem cells. *Cell Transplant* 2013;**22**:1011–1021.

84 I. Cerrada, A. Ruiz-Sauri, R. Carrero, *et al*. Hypoxia-inducible factor 1α contributes to cardiac healing in mesenchymal stem cells-mediated cardiac repair. *Stem Cells Dev* 2013;**22**:501–511.

85 Y. Wang, A. M. Abarbanell, J. L. Herrmann, *et al*. TLR4 inhibits mesenchymal stem cell (MSC) STAT3 activation and thereby exerts deleterious effects on MSC-mediated cardioprotection. *PLoS ONE* 2010;**5**:e14206.

86 J. L. Herrmann, Y. Wang, A. M. Abarbanell, *et al*. Preconditioning mesenchymal stem cells with transforming growth factor-alpha improves mesenchymal stem cell-mediated cardioprotection. *Shock* 2010;**33**:24–30.

87 J. Cho, P. Zhai, Y. Maejima, and J. Sadoshima. Myocardial injection with GSK-3β-overexpressing bone marrow-derived mesenchymal stem cells attenuates cardiac dysfunction after myocardial infarction. *Circ Res* 2011;**108**:478–489.

88 X. Yu, C. Lu, H. Liu, *et al*. Hypoxic preconditioning with cobalt of bone marrow mesenchymal stem cells improves cell migration and enhances therapy for treatment of ischemic acute kidney injury. *PLoS ONE* 2013;**8**:e62703.

89 Z. Pasha, Y. Wang, R. Sheikh, *et al*. Preconditioning enhances cell survival and differentiation of stem cells during transplantation in infarcted myocardium. *Cardiovasc Res* 2008;**77**:134–142.

90 D. Huang, Z. Zhang, B. Chen, *et al*. Therapeutic efficacy of lentiviral vector mediated BDNF gene-modified MSCs in cerebral infarction. *Sheng Wu Gong Cheng Xue Bao* 2008;**24**:1174–1179 (in Chinese).

91 X. Sun, B. Fang, X. Zhao, *et al*. Preconditioning of mesenchymal stem cells by sevoflurane to improve their therapeutic potential. *PLoS ONE* 2014;**9**:e90667.

92 K. H. Jung, D. M. Han, S. G. Jeong, *et al*. Proteomic analysis reveals KRIT1 as a modulator for the antioxidant effects of valproic acid in human bone-marrow mesenchymal stromal cells. *Drug Chem Toxicol* 2015;**38**:286–292.

93 L. Chen, X. Cui, Z. Wu, *et al*. Transplantation of bone marrow mesenchymal stem cells pretreated with valproic acid in rats with an acute spinal cord injury. *Biosci Trends* 2014;**8**:111–119.

94 S. P. Hung, M. H. Yang, K. F. Tseng, and O. K. Lee. Hypoxia-induced secretion of TGF-β1 in mesenchymal stem cell promotes breast cancer cell progression. *Cell Transplant* 2013;**22**:1869–1882.

95 H. M. Ning, J. G. Jin, J. W. Hu, *et al*. Effect of human bone marrow mesenchymal stem cell on allogeneic T lymphocyte phenotype *in vitro*. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2005;**13**:43–49 (in Chinese).

96 M. Li, L. Yu, T. She, *et al*. Astragaloside IV attenuates toll-like receptor 4 expression via NF-κB pathway under high glucose condition in mesenchymal stem cells. *Eur J Pharmacol* 2012;**696**:203–209.

97 H. Y. Chen, X. Zhang, S. F. Chen, *et al*. The protective effect of 17β-estradiol against hydrogen peroxide-induced apoptosis on mesenchymal stem cell. *Biomed Pharmacother* 2012;**66**:57–63.

98 W. Zhang, X. Su, Y. Gao, *et al*. Berberine protects mesenchymal stem cells against hypoxia-induced apoptosis *in vitro*. *Biol Pharm Bull* 2009;**32**:1335–1342.

99 L. Chen, F. J. Cheng, Q. H. Liu, *et al*. Mechanism of granulocyte colony-stimulating factor for promoting cell viability of bone marrow mesenchymal stem cells. *Sheng Li Xue Bao* 2009;**61**:169–174.

100 Y. Kamada, Y. Yoshida, Y. Saji, *et al*. Transplantation of basic fibroblast growth factor-pretreated adipose tissue-derived stromal cells enhances regression of liver fibrosis in mice. *Am J Physiol Gastrointest Liver Physiol* 2009;**296**:G157–G167.

101 J. C. Tille, J. Wood, S. J. Mandriota, *et al*. Vascular endothelial growth factor (VEGF) receptor-2 antagonists inhibit VEGF- and basic fibroblast growth factor-induced angiogenesis *in vivo* and *in vitro*. *J Pharmacol Exp Ther* 2001;**299**:1073–1085.

102 T. J. Poole, E. B. Finkelstein, and C. M. Cox. The role of FGF and VEGF in angioblast induction and migration during vascular development. *Dev Dyn* 2001;**220**:1–17.

103 L. Liu, J. Gao, Y. Yuan, *et al*. Hypoxia preconditioned human adipose derived mesenchymal stem cells enhance angiogenic potential via secretion of increased VEGF and bFGF. *Cell Biol Int* 2013;**37**:551–560.

104 N. Di Maggio, A. Mehrkens, A. Papadimitropoulos, *et al*. Fibroblast growth factor-2 maintains a niche-dependent population of self-renewing highly potent non-adherent mesenchymal progenitors through FGFR2c. *Stem Cells* 2012;**30**:1455–1464.

105 W. T. Lai, V. Krishnappa, and D. G. Phinney. Fibroblast growth factor 2 (Fgf2) inhibits differentiation of mesenchymal stem cells by inducing Twist2 and Spry4, blocking extracellular regulated kinase activation, and altering Fgf receptor expression levels. *Stem Cells* 2011;**29**:1102–1111.

106 S. Tsutsumi, A. Shimazu, K. Miyazaki, *et al*. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun* 2001;**288**:413–419.

107 D. A. Rider, C. Dombrowski, A. A. Sawyer, *et al*. Autocrine fibroblast growth factor 2 increases the multipotentiality of human adipose-derived mesenchymal stem cells. *Stem Cells* 2008;**26**:1598–1608.

108 C. Dombrowski, T. Helledie, L. Ling, *et al*. FGFR1 signaling stimulates proliferation of human mesenchymal stem cells by inhibiting the cyclin-dependent kinase inhibitors p21(Waf1) and p27(Kip1). *Stem Cells* **31**:2724–2736.

109 Y. W. Eom, J. E. Oh, J. I. Lee, *et al*. The role of growth factors in maintenance of stemness in bone marrow-derived mesenchymal stem cells. *Biochem Biophys Res Commun* 2014;**445**:16–22.

110 M. Krampera, L. Cosmi, R. Angeli, *et al*. Role for interferon-γ in the immunomodulatory activity of human bone marrow mesenchymal stem cells. *Stem Cells* 2006;**24**:386–398.

111 J. Croitoru-Lamoury, F. M. Lamoury, M. Caristo, *et al*. Interferon-γ regulates the proliferation and differentiation of mesenchymal stem cells via activation of indoleamine 2,3 dioxygenase (IDO). *PLoS ONE* 2011;**6**:e14698.

112 G. Gastl and C. Huber. The biology of interferon actions. *Blut* 1988;**56**:193–199.

113 A. Meager. Biological assays for interferons. *J Immunol Methods* 2002;**261**:21–36.

114 G. Duque, D. C. Huang, M. Macoritto, *et al*. Autocrine regulation of interferon γ in mesenchymal stem cells plays a role in early osteoblastogenesis. *Stem Cells* 2009;**27**:550–558.

115 C. Vidal, W. Li, B. Santner-Nanan, *et al*. The kynurenine pathway of tryptophan degradation is activated during osteoblastogenesis. *Stem Cells* 2015;**33**:111–121.

116 M. E. Bernardo and W. E. Fibbe. Mesenchymal stromal cells: sensors and switchers of inflammation. *Cell Stem Cell* 2013;**13**:392–402.

117 F. Liotta, R. Angeli, L. Cosmi, *et al*. Toll-like receptors 3 and 4 are expressed by human bone marrow-derived mesenchymal stem cells and can inhibit their T-cell modulatory activity by impairing Notch signaling. *Stem Cells* 2008;**26**:279–289.

118 H. Hwa Cho, Y. C. Bae, and J. S. Jung. Role of toll-like receptors on human adipose-derived stromal cells. *Stem Cells* 2006;**24**:2744–2752.

119 H. S. Kim, T. H. Shin, S. R. Yang, *et al*. Implication of NOD1 and NOD2 for the differentiation of multipotent mesenchymal stem cells derived from human umbilical cord blood. *PLoS ONE* 2010;**5**: e15369.

120 E. Lombardo, O. DelaRosa, P. Mancheño-Corvo, *et al*. Toll-like receptor-mediated signaling in human adipose-derived stem cells: implications for immunogenicity and immunosuppressive potential. *Tissue Eng Part A* 2009;**15**:1579–1589.

121 G. Raicevic, R. Rouas, M. Najar, *et al*. Inflammation modifies the pattern and the function of toll-like receptors expressed by human mesenchymal stromal cells. *Hum Immunol* 2010;**71**:235–244.

122 N. N. Nørgaard, T. Holien, S. Jonsson, *et al*. CpG-oligodeoxynucleotide inhibits Smad-dependent bone morphogenetic protein signaling: effects on myeloma cell apoptosis and *in vitro* osteoblastogenesis. *J Immunol* 2010;**185**:3131–3139.

123 J. Lei, Z. Wang, D. Hui, *et al*. Ligation of TLR2 and TLR4 on murine bone marrow-derived mesenchymal stem cells triggers differential effects on their immunosuppressive activity. *Cell Immunol* 2011;**271**:147–156.

124 M. Pevsner-Fischer, V. Morad, M. Cohen-Sfady, *et al*. Toll-like receptors and their ligands control mesenchymal stem cell functions. *Blood* 2007;**109**:1422–1432.

125 C. A. Opitz, U. M. Litzenburger, C. Lutz, *et al*. Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-β and protein kinase R. *Stem Cells* 2009;**27**:909–919.

126 J. T. Chang, V. R. Palanivel, I. Kinjyo, *et al*. Asymmetric T lymphocyte division in the initiation of adaptive immune responses. *Science* 2007;**315**:1687–1691.

127 G. Vahedi, A. C. Poholek, T. W. Hand, *et al*. Helper T-cell identity and evolution of differential transcriptomes and epigenomes. *Immunol Rev* 2013;**252**:24–40.

128 B. Sacchetti, A. Funari, S. Michienzi, *et al*. Self-renewing osteoprogenitors in bone marrow sinusoids can organize a hematopoietic microenvironment. *Cell* 2007;**131**:324–336.

129 J. Matsui, T. Wakabayashi, M. Asada, *et al*. Stem cell factor/c-kit signaling promotes the survival, migration, and capillary tube formation of human umbilical vein endothelial cells. *J Biol Chem* 2004;**279**:18600–18607.

130 X. Liu, F. Liang, J. Yang, *et al*. Effects of stromal cell derived factor-1 and CXCR4 on the promotion of neovascularization by hyperbaric oxygen treatment in skin flaps. *Mol Med Rep* 2013;**8**:1118–1124.

131 H. P. Gerber and N. Ferrara. The role of VEGF in normal and neoplastic hematopoiesis. *J Mol Med (Berl)* 2003;**81**:20–31.

132 K. A. Moore and I. R. Lemischka. "Tie-ing" down the hematopoietic niche. *Cell* 2004;**118**:139–140.

133 L. C. van den Berk, B. J. Jansen, K. G. Siebers-Vermeulen, *et al*. Mesenchymal stem cells respond to TNF but do not produce TNF. *J Leukoc Biol* 2010;**87**:283–289.

134 A. I. Hoch, B. Y. Binder, D. C. Genetos, and J. K. Leach. Differentiation-dependent secretion of proangiogenic factors by mesenchymal stem cells. *PLoS ONE* 2012;**7**:e35579.

135 A. D. Berendsen and B. R. Olsen. How vascular endothelial growth factor-A (VEGF) regulates differentiation of mesenchymal stem cells. *J Histochem Cytochem* 2014;**62**:103–108.

136 Y. Liu, A. D. Berendsen, S. Jia, *et al*. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. *J Clin Invest* 2012;**122**:3101–3113.

137 K. C. Russell, D. G. Phinney, M. R. Lacey, *et al*. *In vitro* high-capacity assay to quantify the clonal heterogeneity in trilineage potential of mesenchymal stem cells reveals a complex hierarchy of lineage commitment. *Stem Cells* 2010;**28**:788–798.

138 A. Muraglia, R. Cancedda, and R. Quarto. Clonal mesenchymal progenitors from human bone marrow differentiate *in vitro* according to a hierarchical model. *J Cell Sci* 2000;**113**(Pt 7):1161–1166.

139 C. Halleux, V. Sottile, J. A. Gasser, and K. Seauwen. Multi-lineage potential of human mesenchymal stem cells following clonal expansion. *J Musculoskelet Neuronal Interact* 2001;**2**:71–76.

140 T. Okamoto, T. Aoyama, T. Nakayama, *et al*. Clonal heterogeneity in differentiation potential of immortalized human mesenchymal stem cells. *Biochem Biophys Res Commun* 2002;**295**:354–361.

141 K. C. Russell, M. R. Lacey, J. K. Gilliam, *et al*. Clonal analysis of the proliferation potential of human bone marrow mesenchymal stem cells as a function of potency. *Biotechnol Bioeng* 2011;**108**:2716–2726.

142 X. Wang, Q. Cheng, L. Li, *et al*. Toll-like receptors 2 and 4 mediate the capacity of mesenchymal stromal cells to support the proliferation and differentiation of $CD34^+$ cells. *Exp Cell Res* 2012;**318**:196–206.

143 D. Wang, H. Zhang, J. Liang, *et al*. Allogeneic mesenchymal stem cell transplantation in severe and refractory systemic lupus erythematosus: 4 years experience. *Cell Transplant* 2013;**22**:2267–2277.

# Glossary

Note that the position on this list for an abbreviation starting with a numeral will occur in this list according to the numeral, not the word after the numeral

| | |
|---|---|
| 1-MT: | 1-methyl-tryptophan |
| 2D: | two-dimensional |
| 2-DE: | two-dimensional gel electrophoresis |
| 3D: | three-dimensional |
| 3T3: | The 3T3 cell line has become the standard murine fibroblast cell line and was originally established from primary mouse embryonic fibroblast cells. The "3T3" designation refers to the abbreviation of "3-day transfer, inoculum $3 \times 10^5$ cells" |
| 3T3-L1: | a cell line derived from mouse 3T3 cells that is used in biological research on adipose tissue |
| 5caC: | 5-carboxylcytosine |
| 5-FC: | 5-fluorocytosine |
| 5fC: | 5-formylcytosine |
| 5-FU: | 5-fluorouracil |
| 5hmc: | 5 hydroxymethylation |
| 5-HT: | 5-hydoxytryptamine (serotonin) |
| 6MWD: | 6-min walk distance |
| 6-OHDA: | 6-hydroxydopamine |
| 7-ADD: | 7-aminoactinomycin D |
| 14*S*,21*R*-diHDHA: | 14*S*,21*R*-dihydroxydocosa-4*Z*,7*Z*,10*Z*,12*E*,16*Z*,19*Z*-hexaenoic acid |
| 18-F-FDG: | 2-deoxy-2-($^{18}$F)fluoro-D-glucose, a glucose analog with the positron-emitting radioactive isotope fluorine-18 substituted for the normal hydroxyl group at the 2′ position in the glucose molecule for use in PET scanning |
| $^{99m}$Tc: | technetium sestamibi |
| 53BP1: | p53 binding protein 1 |
| $^{111}$In: | indium-111 |
| $^{131}$I FIAU: | $^{131}$I-5-iodo-2-fluoro-1-β-D-arabinofuranosyluracil |
| A2AR: | adenosine A2A receptors |
| A549 cells: | human adenocarcinoma alveolar basal epithelial cells. The A549 cell line was first developed in 1972 by D. J. Giard through the removal and culturing of cancerous lung tissue in the explanted tumor a of 58-year-old Caucasian male |
| AA: | arachidonic acid |
| AAMP: | angio-associated, migratory cell protein |
| AAV: | adeno-associated virus |
| ABPI: | ankle brachial pressure index |
| AC: | articular chondrocyte |
| ACAN: | aggrecan |
| ACE: | angiotensin converting enzyme |
| ACI: | autologous chondrocyte implantation |
| ACL: | anterior cruciate ligament |
| Ac-LDL: | acetylated low-density lipoprotein |
| ACTA2: | alpha-actin-2, also known as actin, aortic smooth muscle, or alpha smooth muscle actin (α-SMA, α-smooth muscle actin) |
| ACTH: | adrenocorticotropic hormone |
| ActR: | activin receptor |
| AD: | Alzheimer's disease[1] |
| ADAM: | a disintegrin and metalloproteinase |
| ADAS: | adipose-derived adult stem cell |
| ADASC: | adipose-derived adult stromal cell |
| ADAMTS5: | a disintegrin and metalloproteinase with thrombospondin motif 5 |
| ADAS: | adipose-derived adult stem cell |
| ADCC: | antibody-dependent cellular cytotoxicity |
| ADDI: | adipocyte determination and differentiation-dependent factor |

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ADM: adrenomedullin
ADMSC or AD-MSC: adipose-tissue-derived mesenchymal stromal cell
AdMSC: adipose mesenchymal stem cell
ADP: adenosine diphosphate
ADSC: adipose-derived stromal cell
AEC: amniotic epithelial cell
AF: amniotic fluid
AFC: alveolar fluid clearance
a-FGF: acidic fibroblast growth factor
AFSC: amniotic fluid stem cell
AHR: airway hyperresponsiveness
AhR: aryl hydrocarbon receptor
AICD: activation-induced cell death
AIE: aggregation-induced emission
AIF: allograft inflammatory factor
AIRE: autoimmune regulator
AKI: acute kidney injury
AKT: the "Ak" in Akt was a temporary classification name for a mouse that developed spontaneous thymic lymphomas. The "t" stands for thymoma. AKT is also known as protein kinase B and is one of the serine/threonine kinases that contain SH2 (Src homology 2-like) domains
ALCAM: activated leukocyte cell adhesion molecule (CD166)
ALDH: aldehyde dehydrogenase
ALI: acute lung injury
ALK: activin-like kinase
ALK5: activin receptor-like kinase 5
ALL: acute lymphoblastic leukemia
ALP: alkaline phosphatase
alpha-SMA: alpha smooth muscle actin
alpha-SMACreERT2: α-smooth muscle actin promoter-directed Cre recombinase estrogen receptor ligand-binding domains
ALPL: alkaline phosphatase
ALPS: autoimmune lympho-proliferative syndrome
ALS: amyotrophic lateral sclerosis
ALT: alanine transaminase
Alu: a short repetitive stretch of primate DNA originally characterized by the action of the Alu (*Arthrobacter luteus*) restriction endonuclease
AMI: acute myocardial infarction
AML: acute myeloid leukemia
AMOC1: SPARC-related modular calcium-binding protein 1
AMOP: adhesion-associated domain in MUC4 and other proteins
AM-MSC: amnion-derived mesenchymal stromal cell
AM-MSC: amniotic-membrane-derived mesenchymal stromal cell
AMP: adenosine monophosphate
Ang: angiopoietin
ANGPTL4: angiopoietin-related protein 4
AOC3: amine oxidase, copper containing 3 (also known as vascular endothelial protein 1
AP-1: activator protein 1
AP2: adipocyte protein 2
AP-2-α: activating enhancer binding protein 2-α
Apaf-1: apoptotic protease-activating factor 1
APC: activated protein C
APC: antigen-presenting cell
APC: colorectal adenoma like protein
APC: adenomatosis polyposis coli (gene/protein)
API: active pharmaceutical ingredient
APN: alanine aminopeptidase
ARCO: Association Research Circulation Osseous (staging system for osteonecrosis)
ARDS: acute respiratory distress syndrome
ARF locus: alternate reading frame protein product of the cyclin-dependent kinase locus
ARGB: Australian Regulatory Guidelines for Biologicals
ARHGEF19: Ras homolog gene family member A guanine nucleotide exchange factor 19
ARNT: aryl hydrocarbon receptor nuclear translocator
ARPE-19: a spontaneously arising retinal pigment epithelial cell line derived in 1986 by Amy Aotaki-Keen from the normal eyes of a 19-year-old male
ARTG: Australian Register of Therapeutic Goods
AS: antisense
ASC: adipose-derived stromal cell
ASC: adipose-derived stem/stromal cell
ASC: acellular spinal cord

ASIA: American Spinal Injury Association (scoring system for spinal cord injury)
AST: aspartate aminotransferase
ATF4: activating transcription factor 4
ATG: antithymocyte globulin
ATM: adipose tissue macrophages
ATM: ataxia-telangiectasia-mutated
ATMP: advanced therapy medicinal product
AT-MSC: adipose-tissue-derived mesenchymal stromal cell
ATP: adenosine-5–triphosphate
ATR: ATM and RAD3 related
B7 DC: B7 dendritic cell DC (also known as PD-L2, CD273)
B7 H1: B7 homolog (also known as PD-L1 and CD274)
B7-1: CD80
B7-2: CD86
BAD: BCL-XL/BCL2-associated death promoter
BAFF: B cell activating factor
BAL: bronchoalveolar lavage fluid
BALP: bone-specific alkaline phosphatase
BARK: β-adrenergic receptor kinase
BAX: Bcl-2-associated X protein
BBB: blood–brain barrier
BBB: Basso, Beattie, and Bresnahan scoring system for assessing locomotor recovery
BCG: Bacille de Calmette et Guérin (bacillus of Calmette and Guerin)
BCL-2: B cell lymphoma-2
BCL-XL: B cell-lymphoma extra large
BCR: B cell receptor
BCR–ABL: the fusion gene of the break point cluster (BCR) gene on human chromosome 22 with the Abelson (ABL) tyrosine kinase gene on human chromosome 9, resulting in the chimeric oncogene BCR–ABL
BDKRB1: bradykinin receptor B1
BDNF: brain-derived neurotrophic factor
beta2-AR: β-2 adrenergic receptor
beta-2-AR: β-2 adrenergic receptor
beta-NGF: β-nerve growth factor
bFGF: basic fibroblast growth factor
BGN: biglycan
β-GP: β-glycerophosphate sodium
BH: Bcl-2 homology
bHLH: basic-helix–loop–helix
BIL: bioluminescence imaging
BIO: indirubin 6-bromoindirubin-3′-oxime
BIRC8: baculoviral inhibitor of apoptosis repeat containing protein 8
BLI: bioluminescent imaging
BLM: Bloom syndrome (helicase)
Blnc1: brown adipose tissue long noncoding RNA 1
BM: basement membrane
BM: bone marrow
BMC: bone mineral content
BMD: bone mineral density
BMI: body mass index
BM-MNC: bone marrow mononuclear cell
BM-MSC: bone-marrow-derived mesenchymal stromal cell
BMSC: bone marrow stromal cells
BMP: bone morphogenetic protein
BMPR1A: bone morphogenetic protein receptor 1A
BMT: bone marrow transplantation
BODIPY: boron-dipyrromethene (a lipid probe)
BOOST: BOne marrOw transfer to enhance ST-elevation infarct regeneration (name given to a clinical trial for acute myocardial infarction
BOS: bronchiolitis obliterans syndrome
bp: base pair
BPD: bronchopulmonary dysplasia
Br1: B regulatory cells 1
BRCA1: breast cancer type 1
BRCT: breast cancer type 1 C-terminal (domains)
BrdU: 5-bromo-2′-deoxyuridine
Bregs: B regulatory cells
BSA: bovine serum albumin
BSC: bulge stem cells (of hair follicles)
BSMC: bronchial smooth muscle cells
BSP: bone sialoprotein
BTB: blood–tumor barrier
BV/TV: bone volume/trabecular volume
bZIP: basic region/leucine zipper domain
CA1P: combretastatin A1 diphosphate, also known as OXi4503 (a dual-mechanism vascular disrupting agent)

CABG: coronary artery bypass grafting
CAD: coronary artery disease
CAD: caspase-activated DNAse
CAD: calcium-dependent adhesion
CAF: cancer-associated fibroblast
CALLA: common acute lymphoblastic leukemia antigen
CAM: chick embryo chorioallantoic membrane
CAM: cell adhesion molecule
CAMKII: calcium/calmodulin-dependent kinase II
cAMP: cyclic adenosine monophosphate – a second messenger important in many biological processes; cAMP is derived from adenosine triphosphate and is used for intracellular signal transduction in many different organisms, conveying the cAMP-dependent pathway
CAR: CXCL12-abundant reticular (stromal cell)
CARD: caspase activation and recruitment domain
CASP: colon ascendens stent peritonitis
CBER: Center for Biologics Evaluation and Research (USA)
CBFA1: core-binding factor alpha 1 (another name for RUNX2)
CBP: phosphorylated CREB-binding protein
CBV: cerebral blood volume
CCAAT: cytosine–cytosine–adenosine–adenosine–thymidine
CCD: charge-coupled devices
cCFU-F: cardiac colony-forming unit–fibroblastic
CCL: chemokine (C–C motif) ligand
CCL4: carbon tetrachloride
CCL5: chemokine (C–C motif) ligand 5. It is also known as RANTES
CCM: concentrated conditioned medium
CCR: chemokine receptor
CCSP: Clara cell secretory protein
CCTRN: Cardiovascular Cell Therapy Research Network
CD: cluster of differentiation
CD: Crohn's disease
CD: cytosine deaminase
CDAI: Crohn's disease activity index
CDC: cell division control (protein)
CDEIS: simple endoscopic score for Crohn's disease
CDER: Center for Drugs Evaluation and Research (USA)
CDH5: cadherin 5, type 2 (vascular endothelium)
CDK: cyclin-dependent kinase
cDNA: complementary DNA (double-stranded DNA synthesized from a messenger RNA template by reverse transcriptase)
CdSe–ZnS QDs: quantum dots composed of dimethylcadmium, diethylzinc, and selenide
C/EBP: CCAAT-enhancer binding protein
C/EBP-α: CCAAT-enhancer-binding protein-α
C/EBP-β: CCAAT/enhancer-binding protein β
CFDA-SE: carboxyfluorescein diacetate succinimidyl ester
CFP: cyan fluorescent protein
CFSE: carboxyfluorescein *N*-hydroxysuccinimidyl ester (a cell permeable, nonfluorescent pro-dye)
CFR: Code of Federal Regulations (USA FDA)
CFU-A: colony forming unit–adipocyte
CFU-F: colony-forming unit–fibroblast
CFU-GM: colony-forming units of granulocyte and macrophage
CFU-O: colony forming unit–osteoblast
CFU-S: colony-forming unit–spleen
cGMP: current Good Manufacturing Practice
cGMP: cyclic guanosine monophosphate
CGRP: calcitonin gene related peptide
CGTP: Current Good Tissue Practices
CHAD: chondroadherin
ChIP: chromatin immunoprecipitation
ChIP-Seq: chromatin immunoprecipitation sequencing
CHK: checkpoint kinase
CIA: collagen-induced arthritis
CIF: cumulative incidence functions
CII2A: (MHC) class II transactivator
CINC: cytokine-induced neutrophil chemoattractant (also known as GRO)
CK: cytokeratin

CK2: checkpoint kinase 2
CKD: chronic kidney disease
CKI: CDK inhibitor
CLI: critical limb ischemia
CLP: cecal ligation and puncture
CLP: committed lymphoid progenitor
CLR: C-type lectin receptor
CM: conditioned medium
c-MAF: musculoaponeurotic fibrosarcoma oncogene homolog
CMB: condensed mesenchymal bodies
C-MSC: chorion-derived mesenchymal stromal cell
CNI: calcineurin inhibitor
CNN: this refers to the CCN family of extracellular matrix-associated signaling proteins (also known as matricellular proteins). The acronym CCN is derived from the first three members of the family discovered, namely CYR61 (cysteine-rich angiogenic protein 61 or CCN1), CTGF (connective tissue growth factor or CCN2), and NOV (nephroblastoma overexpressed or CCN3). Together with three Wnt-induced secreted proteins, they comprise the CCN family of matricellular proteins. These proteins have now been renamed CCN1–6 by international consensus
CNPase: 2′,3′-cyclic-nucleotide 3′-phosphodiesterase
CNS: central nervous system
CNTF: ciliary neurotrophic factor
CO: cardiac output
COAD: chronic obstructive airway disease
$CoCl_2$: cobalt chloride
COL1: collagen type 1
COL1A1: collagen type 1, alpha 1
COMP: cartilage oligomeric matrix protein
COPD: chronic obstructive pulmonary disease
COS: a fibroblast-like cell line derived from monkey kidney tissue. COS cells are obtained by immortalizing CV-1 cells with a version of the SV40 virus that can produce large T antigen but has a defect in genomic replication. The CV-1 cell line in turn was derived from the kidney of the African green monkey. The acronym "COS" is derived from the cells being CV-1 (simian) in Origin, and carrying the SV40 genetic material
COUP-TFII: chicken ovalbumin upstream promoter transcription factor II
COX: cyclooxygenase
CpG: cytosine–phosphate–guanosine
CR: chromatin remodeler
CR: competing risks
CR3: complement receptor 3
CRAC1: cartilage acidic protein 1
CRADs: conditionally-replicating adenoviruses
CRE: Cre protein encoded by the locus originally named as "Causes recombination" with "cyclization recombinase"
CRE: cAMP response element
CREB: cyclic AMP response element binding protein
CRP: C-reactive protein
CSC: cardiac stem cell
CSC: cancer stem cells
CSF: cerebrospinal fluid
CSF-1: colony stimulating factor 1 (also known as M-CSF)
CSP: cyclosporine (also known as cyclosporin and cyclosporine A)
CSPG: chondroitin sulfate proteoglycan
CT: computed tomography
CTB: C-terminus binding protein
CTB: cholera toxin-B chain
CTGF: connective tissue growth factor
CTiP: C-terminus interacting protein
CTL: cytotoxic T lymphocyte
CTLA-4: cytotoxic T-lymphocyte-associated protein 4, also known as CD152
CTC: chorionic trophoblastic cells
CTX: C-telopeptide
CTX: cytoxan (cyclophosphamide)
CVA: cerebrovascular accident
CVD: cardiovascular disease
CVD: cerebrovascular disease
CV-MSC: chorionic villi-derived mesenchymal stromal cell
CX3CL1: C–X–C motif chemokine ligand 3 (also known as fraktalkine)
CX43: connexin 43
CXCL12: C–X–C motif chemokine ligand 12

CXCR4: C–X–C motif chemokine receptor 4
CYP3A4: cytochrome P450 3A4
Cyr61: cysteine-rich angiogenic inducer 61
DA: dopaminergic
DAF: decay-accelerating factor
DAMP: damage-associated molecular pattern
DAP 10: DNAX-activation protein 10
DAPI: 4′,6-diamidino-2-phenylindole, a fluorescent stain that binds strongly to A–T-rich regions in DNA and is often used to stain the nucleus of cells
dATP: deoxyadenosine triphosphate
DAPT: *N*-[*N*-(3, 5-difluorophenacetyl-L-alanyl)]-(*S*)-phenylglycine *t*-butyl ester
DC: dendritic cell
DC: direct current
DCN: decorin
DD: death domain
DDR: DNA damage response
DES: desmin
DETA NONOate: (*Z*)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl)amino]diazen-1-ium-1,2-diolate
DEXA: dual-energy X-ray absorptiometry
DFX: desferrioxamine
DIC: differential interference contrast (microscopy)
DiI: a chemical that is a fluorescent lipophilic cationic indocarbocyanine dye. The "Di" possibly stands for the di-alkyl nature and the "I" of DiI possibly stands for the "indocarbocyanine" group
DISC: death-inducing signaling complex
DKK1: Dickkopf factor
DLA: dog leukocyte antigen
DLCO: diffusing capacity for carbon monoxide
DLL4: delta like ligand
Dll4: delta-like ligand 4
DLX: Distal-less homolog
DMEM: Dulbecco's modified Eagle medium[2,3]
DMP1: dentin matrix protein 1
D-MSC: decidua-derived mesenchymal stromal cell
dMTase: DNA demethylases
DNA: deoxyribonucleic acid
DNAX: the gene that codes for the τ and γ subunits of the DNA clamp loader of prokaryotes. The eukaryotic equivalent to the DNA clamp loader is Replication Factor C, which is encoded by the genes RFC1, RFC2, RFC3, RFC4, and RFC5
DNA-PKcs: DNA-dependent protein kinase, catalytic subunit
DNMT: DNA methyltransferase
DNPEP: aspartyl aminopeptidase
dNPT: deoxyribonucleotide
DPSC: dental pulp stem cell
DSB: double-strand break
dsRNA: double-stranded RNA
DSS: dextran sodium sulfate
DTR: diphtheria toxin receptor
Dvl: Dishevelled (protein)
E1A: early 1A adenoviral (gene or protein)
EAE: experimental autoimmune encephalitis
EB: embryoid body
EBF2: early B cell factor 2
EBMT: European Group for Blood and Marrow Transplantation
E-cadherin: epithelial cadherin (cadherin = calcium-dependent adhesion)
ECD: expanded criteria donor
ECE: endothelin converting enzyme
ECFR: epidermal growth factor receptor
ECM: extracellular matrix
EDF-1: endothelial differentiation-related factor 1
EDP: end-diastolic pressure
EdU: 5-ethynyl-2′-deoxyuridine
EDV: end-diastolic volume
EEF1A1: eukaryotic translation elongation factor 1A1
EEV: endothelial extracellular vesicle
EFEMP: EGF-containing fibulin-like extracellular matrix protein
EGF: epidermal growth factor
eGFP: enhanced green fluorescent protein
EGFRvIII: epidermal growth factor receptor variant III
EGM: endothelial cell growth medium
EHS: Engelbreth-Holm–Swarm (Type of mouse sarcoma cell that secretes a gelatinous protein mixture marketed as Matrigel. The chief components of Matrigel are structural proteins such

as laminin, entactin, collagen, and heparan sulfate proteoglycans that present cultured cells with the adhesive peptide sequences that they would encounter in their natural environment. Also present are growth factors like TGF-β and EGF that prevent differentiation and promote proliferation of many cell types)

EIA: Adenovirus early region 1A (E1a or E1A), (a gene expressed during adenovirus replication to produce a variety of (E1A) proteins)

EIA: E1A binding protein p300, also known as EP300 or p300, is a protein that, in humans, is encoded by the EP300 gene also known as EID-1

EID-1: the gene that encodes EP300-interacting inhibitor of differentiation 1

ELF: enzyme-labeled fluorescence

ELISA: enzyme-linked immunosorbent assay

ELN: elastin

EMA: European Medicines Agency

eMSC: endometrial MSC

EMT: epithelial-to-mesenchymal transition

ENaC: alfa-epithelial sodium channel

EndMT: endothelial-to-mesenchymal transition

eNOS: endothelial nitrous oxide synthase

EP2: receptor for prostaglandin $E_2$

EP4: receptor for prostaglandin $E_2$ subtype EP4

EPC: endothelial progenitor cell

EPO: erythropoietin

EPOR: erythropoietin receptor

ER: endoplasmic reticulum

ERC: endometrial regenerative cells

ERK: extracellular signal-regulated kinase

ERM: the ERM protein family consists of the three closely related proteins ezrin, radixin, and moesin

ESC: embryonic stem cell

ESCRT: endosomal sorting complex required for transport

ESRD: end-stage renal disease

ESV: end-systolic volume

ET-1: endothelin-1

ETA-1: early T-cell activation factor 1

ETS: E26 transformation-specific or E-twenty-six (a member of one of the largest families of transcription factors)

ETSV1: E (twenty-six) family of transcription factors variant 1

EU: European Union

EV: extracellular vesicles

EYA1/3 eyes absent homolog 1/3 (phosphatase)

EZH2: Enhancer of Zeste homolog two

EZR: ezrin

FA: fatty acid

FABP4: fatty acid binding protein 4

FACS: fluorescence-activated cell sorting

FADD: Fas-associated protein with death domain

FAP: fibroblast activation protein

FAK: focal adhesion kinase

FAP-α: fibroblast activation protein-α

FAS: it is not clear for what FAS is an abbreviation; one suggestion is "first apoptotic signal," since engagement of Fas (a cell death surface receptor) by Fas ligand (FasL) results in apoptotic cell death mediated by caspase activation

FAT: fatty acid translocase (also known as CD36)

FBA: fetal bovine albumin

FBS: fetal bovine serum

FcεRI: Fc epsilon receptor 1

FCS: fetal calf serum

FDA: Food and Drug Administration (USA)

FEV1: forced expiratory volume in 1 s

FGF: fibroblast-derived growth factor

FGFR: fibroblast growth factor receptor

FIAU: fluoro-1-β-D-arabinofuranosyluracil

FIH: factor inhibiting HIF

FISH: fluorescence *in situ* hybridization

FIX: (coagulation) factor IX

FKBP: FK506 binding protein 5 (FK506 was the initial term used for the calcineurin inhibitor tacrolimus)

Flt: FMS-like tyrosine kinase

FMA: fluorescent migroangiography

f-MLF: formyl–methionyl–leucyl phenylalanine

FMOD: fibromodulin
FMS: factor increasing monocytopoiesis
FN: fibronectin
FN9-10: fibronectin fragment 9–10
FOS: Finkel–Biskis–Jinkins murine osteogenic sarcoma virus (gene, protein)
FPR: *N*-formylated peptides/*N*-formyl peptide receptor
FPRL: formyl peptide receptor-like
FOV: field of view
FOX: forkhead winged helix box
FRAP: fluorescence recovery after photobleaching
FRET: fluorescence energy transfer
F/RGD: a modified adenoviral vector (Adv-F/RGD) with an RGD-containing peptide in the HI loop of the fiber knob domain of adenovirus type 5
FSH: follicle-stimulating hormone
FVC: forced vital capacity
FVIII: (clotting) factor VIII
FZD: Frizzled
FZD8: frizzled class receptor 8
$G_0$: gap 0 (quiescent phase of the cell cycle)
$G_1$: gap 1 (of the cell cycle)
$G_2$: gap 2 (of the cell cycle)
G6PT: glucose-6-phosphate transporter
GABA: γ-aminobutyric acid
GAG: glycosaminoglycan
GALS1: lectin, galactoside-binding, soluble, 1
GA-MSC: glioma-associated MSCs
GAP-43: growth associated protein-43
GAPDH: glyceraldehyde 3-phosphate dehydrogenase
GAS 6: growth arrest-specific gene 6
GATA: GATA transcription factors are a family of transcription factors characterized by their ability to bind to the DNA sequence GATA
GBM: glioblastoma multiforme
GCP: granulocyte chemotactic protein (also known as CXCL6)
GCP: good clinical practice
cGCP: current good clinical practice
G-CSF: granulocyte colony-stimulating factor
GCTB: giant cell tumor of bone
GD2: neural ganglioside-2
GDF5: growth differentiation factor 5
GDNF: glial-derived neurotrophic factor
GDNF: glial-cell-line-derived neurotrophic factor
GEF-H1: guanine nucleotide exchange factor H1
GFAP: glial fibrillary acid protein
GFP: green fluorescent protein
GFR: glomerular filtration rate
GGF: glial growth factor
GGT: γ-glutamyltransferase
GICs: glioma-initiating cells
GJCs: gap junctional channels
Gli: glioma-associated antigen
GLUT: glucose transporter
GMP: good manufacturing practice
GO: gene ontology
GOLD: Global Initiative on Obstructive Lung Disease
GPD1: glycerol-3-phosphate dehydrogenase
GPI: glycophosphatidylinositol
GPR39: G-protein coupled receptor 39
Gr-1: granulocyte receptor-1
GREM1: gremlin 1
GRO: growth-related oncogene
GRO-1α: growth-related protein 1 (also known as CINC, but now known as CXCL1)
GSC: glioma stem cell
GSK: glycogen synthase kinase
GSNOR: *S*-nitrosoglutathione reductase gene
GTP: guanosine triphosphate
GUSB: β-glucuronidase
GUI: graphical user interface
GVHD: graft-versus-host disease
GVL: graft-versus-leukemia (effect)
Gy: gray (a unit of ionizing radiation)
H2: the major histocompatibility complex of mice
$H_2O_2$: hydrogen peroxide
H9C2: a subclone of an original clonal cell line derived from embryonic BD1X rat heart tissue
HA: hyaluronic acid
hAMSC: human amnion-derived MSC
HAND 2: heart- and neural crest derivatives-expressed protein 2

HAPLN 1: hyaluronan and proteoglycan link protein 1
HAS: hyaluronic acid synthase
HA-TCP: hydroxyapatite–tricalcium phosphate
HAT: histone acetyltransferase
HbA1c: glycated hemoglobin
HB-EGF: heparin-binding EGF-like growth factor
HBO: hyperbaric oxygen
HBPA: heparin-binding peptide amphiphile
HCAM: homing cell adhesion molecule (CD44)
HCC: hepatocellular cancer
HCELL: hematopoietic cell E-selectin/L-selectin ligand
hCMSC: human chorion-derived MSC
HCT/P: human cell and tissue products (USA FDA)
HD: Huntington's disease[4]
HDAC: histone deacetylase
HDD: hard disk drives
HDL: high-density lipoprotein
HDM: house dust mite
HDMT: histone demethylase
hDPSC: human dental pulp stem cell
HEK: human embryonic kidney
HeLa: a cell line derived from cervical cancer cells taken on February 8, 1951, from Henrietta Lacks
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (buffer)
HES: human embryonic stem cells
HES1: transcription factor HES1 (hairy and enhancer of split-1)
HEY: hairy/enhancer-of-split related with YRPW motif protein
HGF: hepatocyte growth factor
HGFR: hepatocyte growth factor receptor
HGMA2: high-mobility group AT-hook 2
HIF: hypoxia-inducible factor
HIMF: hypoxia-induced mitogenic factor
HLA: human leukocyte antigen
HLI: hind limb ischemia
HMADS: human multipotent adipose-derived stromal cells
HME: hematopoietic microenvironment
HMEC-1: human microvascular endothelial cell line
HMG-CoA reductase: 3-hydroxy-3-methyl-glutaryl-CoA reductase
HMOX1: heme oxygenase 1
HMPAO: hexamethylpropylene
HMT: histone methyltransferase
HO: heme oxygenase
HOX: homeotic genes encoding the homeobox
hPAP: human placental alkaline phosphatase
HPC: hematopoietic progenitor cell
HPLC: high-performance liquid chromatography
HPMA-RGD: poly[*N*-(2-hydroxypropyl) methacrylamide with the attached amino acid sequence Arg–Gly–Asp] (scaffold)
HR: homologous recombination
H/R: hypoxia and reoxygenation
HREC: human research ethics committee
HSB: hyperactive sleeping beauty (The Sleeping Beauty transposon system is composed of a Sleeping Beauty transposase and a transposon. The resurrected transposase gene was named "Sleeping Beauty" because it was brought back to activity from a long evolutionary sleep)
HSC: hematopoietic stem cell
HSCT: hematopoietic stem cell transplantation
HSP: heat shock protein
HSPC: hematopoietic stem and progenitor cell
HSV-TK: herpes simplex virus thymidine kinase
H-TERT: human telomerase reverse transcriptase
HTT: huntingtin, the gene for Huntington's chorea IT15 (and on initial discovery as IT15)
HU: hydroxyurea (a cytotoxic agent)
HUCPVC: human umbilical cord perivascular cells
HUMSC-NS: human umbilical cord mesenchymal stromal-cell-derived neurosphere
HUVEC: human umbilical vein endothelial cell
IA: intra-arterial
IαI: inter-alpha-inhibitor
IAP: inhibitor of apoptosis
IBA1: ionized calcium-binding adapter molecule (also known as allograft inflammatory factor (AIF))

IBD: inflammatory bowel disease
IBMX: 1-methyl-3-isobutylxanthine
IBZ: ischemic boundary zone
IC: intracranial
ICA: internal carotid artery
ICAM-1: intercellular adhesion molecule 1
ICAT: isotope-coded affinity tagging
ICC: immunocytochemistry
ICC: intercellular communication
ICH: International Conference on Harmonisation
ID1: DNA-binding protein inhibitor
IDO: indoleamine 2,3-dioxygenase
IFATS: International Federation for Adipose Therapeutics and Science
IFATS: International Fat Applied Technology Society
IFN: interferon
IFN-γR: interferon-γ receptor
IGF-1: insulin-like growth factor-1
IGFBP: insulin-like growth factor-binding protein
IGR1R: insulin-like growth factor 1 receptor
IHC: immunohistochemistry
IHH: Indian hedgehog
IκB: inhibitor of kappa light polypeptide gene enhancer in B cells, kinase beta
IκBα: inhibitor of kappa Bα
IKK-β: inhibitor of kappa kinase β
IL: interleukin
IL-12M: modified interleukin-12
IL-1RA: interleukin-1 receptor antagonist
ILB: intraluminal vesicle
ILC2: innate lymphoid cells 2
ILK: integrin-linked kinase
ILT: immunoglobulin-like transcript
ILV: intraluminal vesicles
IMDM: Iscove's modified Dulbecco's medium[5]
IMZ: $^{123}$I-iomazenil
INC: intercellular nanotubes
IND: investigational new drug
INF: interferon
Ink4A: inhibitor of cyclin-dependent kinase 4A
iNOS: inducible nitric oxide synthetase
IP6K: inositol hexakisphosphate kinase
IP 10: interferon-γ-induced protein
IP: ischemic preconditioning
IP: intraperitoneal
IPLASS: International Placenta Stem Cell Society
iPSC: induced pluripotent stem cell
iPSC-MSCs: MSCs derived from induced pluripotent stem cells
IQGAP1: Ras GTPase-activating-like protein
IR: ionizing radiation
IRAK: interleukin-1 receptor-associated kinase
IRIF: ionizing-radiation-induced foci
IRI: ischemia–reperfusion injury
IRS-1: insulin receptor substrate 1
I-SMAD: inhibitor SMAD
ISCT: International Society for Cell Therapy
ITGA11: integrin α11
ITGA2: integrin α2 (also known as CD49B, α2 subunit of the very late activation antigen-2 receptor and platelet membrane glycoprotein Ia (GP1a))
ITGA5: integrin α5 (also known as the fibronectin receptor, CD49e and very late activation protein 5 (VLA-5))
ITGAM: integrin alpha M (also known as CD11b)
ITGB1: integrin β1 (also known as CD29, glycophorin IIa and very late activation protein, β subunit)
ITP: idiopathic thrombocytopenic purpura
iTRAQ: isobaric tagging for relative and absolute quantitation
IV: intravenous
IVI: intravenous infusion
JAG1: jagged 1
JAK: Janus family kinase (not as alleged by some as "just another kinase")
JMJD3: Jumonji domain-containing protein 3
JNK: c-Jun NH2-terminal kinase
JUN: Jun N-terminal kinase
JUND: jun D proto-oncogene
KC: keratinocyte chemoattractant; keratinocyte-derived chemokine
kDa: kilodalton
KDR: kinase insert domain receptor (a type III receptor tyrosine kinase) also known as vascular endothelial growth factor receptor 2 (VEGFR-2)
KGF: keratinocyte growth factor

Ki-67: an antigen indicative of cellular proliferation identified by the monoclonal antibody Ki-67

KID: kinase insert domain receptor (also known as VEGFR)

KIR: killer inhibitory receptor

Kim: kidney injury model

KLF: Kruppel-like factor

KO: knockout

kPa: kilopascal

KSC: keratinocyte stem cells

KT: kidney transplantation

LA-4 cells: cells from a murine urethane-induced lung adenoma

LABA: long-acting beta agonist

LACZ: lactose operon gene Z (encodes β-galactosidase)

LAD: left anterior descending (artery)

LAMPB: lysosomal-associated membrane protein B

LC–MS/MS: liquid chromatography–mass spectrometry/mass spectrometry

LDH: lactic dehydrogenase

LDL: low-density lipoprotein

LEF: lymphoid enhancer-binding factor

LEF/TCF: LEF – see earlier; TCF – transcription factor (A group of transcription factors that bind to DNA through a high-mobility group domain. They are involved in the Wnt signaling pathway, where they recruit the coactivator β-catenin to enhancer elements of genes they target)

LEPR: leptin receptor

LET-7: lethal-7

LEV: lymphocyte extracellular vesicle

LFA-1: lymphocyte function-associated antigen 1 (found on all T-cells and also on B-cells, macrophages and neutrophils and involved in recruitment to the site of infection. It binds to ICAM-1 on antigen-presenting cells and functions as an adhesion molecule)

$Li^+$: lithium ion

LiCl: lithium chloride

LIF: leukemia inhibitory factor

LIM: LIM domains are protein structural domains, composed of two contiguous zinc finger domains, separated by a two-amino-acid residue hydrophobic linker. They are named after their initial discovery in the proteins Lin11, Isl-1, and Mec-3

Lin: lineage

LL37: leucine, leucine-37

LLP2A: *N*-[[4-[[[(2-ethylphenyl)amino] carbonyl]amino]phenyl]acetyl]-Ne-6-[(2*E*)-1-oxo-3-(3-pyridinyl-2-propenyl)]-L-lysyl-L-2-aminohexanedioyl-(1-amino-1-cyclohexane) carboxamide

LncRNAs: long noncoding RNAs

LNGFR: low-affinity nerve growth factor receptor

Lox P: locus of X-over in bacteriophage P1

LPL: lipoprotein lipase

LPS: lipopolysaccharide (endotoxin)

LRP: low-density lipoprotein receptor

LSC: leukemic stem cell

LSCD: limbal stem cell deficiency

LSD1: lysine-specific demethylase 1

L-SOX5: long SRY-related HMG-box 5

LST1: leukocyte-specific transcript

LT: liver transplantation

LT: lymphotoxin

LTA: lymphotoxin-α

LTBP: latent transforming growth factor beta binding protein

LTC4: leukotriene C4

LTQ: linear trap quadrupole

LUM: lumican

LV: left ventricle

L-VAP-2: lymphocyte-vascular adhesion protein 2 (also known as CD73 and 5′-nucleotidase

LVDP: left ventricular diastolic pressure

LVEF: left ventricular ejection fraction

LVF: left ventricular function

LY294002: a morpholine-containing chemical compound that is a potent inhibitor of phosphoinositide 3-kinases (PI3Ks)

LYVE-1: lymphatic vessel endothelial receptor 1

M: mitosis (phase of the cell cycle)

M cells: microfold cells – cells found in the follicle-associated epithelium of the Peyer's patches as well as in bronchus-associated lymphoid tissue (BALT). They transport organisms

| | |
|---|---|
| | and particles from the gut lumen to immune cells across the epithelial barrier and thus are important in stimulating mucosal immunity |
| MAB or mAb: | monoclonal antibody |
| MAC: | membrane attack complex |
| MACI: | matrix-induced chondrocyte implantation |
| MAC-IP: | membrane attack complex inhibitory protein |
| MACS: | magnetic activated cell sorting |
| MadCAM: | mucosal vascular addressin cell adhesion molecule |
| MAEC: | murine aortic endothelial cells |
| MAMP: | microbe-associated molecular pattern |
| MAP: | medroxyprogesterone acetate (also known as 17α-hydroxy-6α-methylprogesterone acetate) |
| MAP: | microtubule-associated protein |
| MAPC: | multipotent adult progenitor cells |
| MAPK: | mitogen-activated protein kinase |
| MAP2K: | mitogen-activated protein kinase kinase (also known as MAPKK and MEK) |
| MAPKK: | mitogen-activated protein kinase kinase (also known as MAPK, MAP2K and MEK) |
| MASC: | multipotent adult mesenchymal stromal cell |
| MATN4: | matrilin 4 |
| MBA: | microbioreactor array |
| MBD: | methyl-CpG-binding domain |
| MBF: | methyl binding factors |
| MBP: | myelin basic protein |
| MCA: | middle cerebral artery |
| MCAM: | melanoma cell adhesion molecule |
| MCAO: | middle cerebral artery occlusion |
| MCK: | muscle creatine kinase |
| MCP-1: | monocyte chemoattractant protein 1 (also known as CCL2) |
| M-CSF: | macrophage colony-stimulating factor (also known as CSF-1) |
| MDC: | macrophage-derived chemokine |
| MDC1: | mediator of DNA damage checkpoint protein 1 |
| MDCT: | multi-detector computed tomography |
| MDFIC: | MyoD family inhibitor domain containing |
| MDHLC: | mesenchymal-derived hepatocyte-like cells |
| Mdm2: | mouse double minute 2 (ligase) |
| MDSC: | muscle-derived stem cell |
| MDSC: | myeloid-derived suppressor cell |
| MeDIP-chip: | methyl-DNA immunoprecipitation and promoter array hybridization |
| meCp2: | methyl CpG binding protein 2 |
| MEF2C: | myocyte enhancer factor 2C |
| MEK: | mitogen/extracellular signal-regulated kinase (also known as MAPK, MAP2K and MAPKK). |
| MEM: | minimum essential medium |
| MEP: | motor-evoked potential |
| MESC: | mouse embryonic stem cell |
| MET: | mesenchymal-to-epithelial transition |
| MEV: | monocyte extracellular vesicle |
| MFGE8: | milk fat globule EGF factor 8 protein |
| mGFP: | membrane-bound GFP |
| mGFP: | monomeric GFP |
| MGP: | matrix gla protein |
| MHC: | major histocompatibility complex |
| MHC: | myosin heavy chain |
| MHRA: | Medicines and Healthcare Products Regulatory Agency |
| mHTT: | mutant huntingtin (gene) |
| μCT: | micro computed tomography |
| MI: | myocardial infarct |
| MIAMI: | marrow-isolated adult multilineage inducible (cells) |
| MICA: | major histocompatibility complex (MHC) class I chain-related A |
| MIF: | macrophage inhibitory protein |
| MIG: | monocyte induced by γ-interferon |
| MIP: | macrophage inflammatory protein |
| MIRL: | membrane inhibitor of reactive lysis |
| miRNA: | microRNA |
| MIRO-1: | mitochondrial Ras homolog gene family member T1 (Rho) GTPase 1 |
| mitoGFP: | GFP that targets mitochondria specifically |
| mitoRFP: | RFP specifically targeting mitochondria |
| MITR: | myocyte enhancer factor-2 interacting transcription factor |
| MKK: | MAPK kinases |
| MKKK: | MAPK kinase kinases |
| MLHFW: | Minnesota Living with Heart Failure (score) |
| MLL1–5: | mixed lineage leukemia 1–5 |

MLR: mixed lymphocyte reaction or mixed leukocyte reaction
MME: metallo-endopeptidase
MMF: mycophenolate mofetil
MMP: matrix metalloproteinase
MMTV: mouse mammary tumor virus
MNC: mononuclear cell
MNP: magnetic nanoparticle
MnSOD: manganese superoxide dismutase
MOE: membrane-organizing extension spike protein
MP: microparticle
MPC: mesenchymal progenitor cell
mpCCL2: matrix metalloproteinase-processed CCL2
MPP: multipotent progenitor
MPSVIII: mucopolysaccharidosis type VII
MR: mannose receptor
MRA: magnetic resonance angiogram
MRI: magnetic resonance imaging
MRN: Mre11-Rad50-Nbs1
mRNA: messenger RNA
MRTF-A: myocardin-related transcription factor-A
MS: mass spectrometry
MSC: mesenchymal stromal cell
MSC1: mesenchymal stromal cell type 1
MSCA-1: mesenchymal stem cell antigen-1
MSC-Ex: mesenchymal stromal cell exosome
MSDNC: marrow stromal-cell-derived neuronal cell
M-SEC: a protein involved in tunneling nanotubules and so called because it was first found in intestinal M (microfold) cells and is homolgous to Seq6, a subunit of the exocyst
MSX2: Msh homeobox protein 2
MSN: medium spiny neuron
MSN: mesoporous silica nanoparticle
M-SPIO: micrometer-sized superparamagnetic iron oxide
MSS: MRI score of severity
MT1-MMP: membrane-associated type-I transmembrane matrix metalloproteinase
MTD: maximum tolerated dose
mtDNA: mitochondrial DNA
mTEC: medullary thymic epithelial cell
MTMMP: membranous type matrix metalloproteinase
mTORi: mammalian target of rapamycin inhibitor
MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUC: mucin
MV: microvesicle
MVB: multivesicular body
MVO2: maximal oxygen consumption
Mx1: a stromal cell line with MSC features that has the myxovirus resistance-1 (Mx1) promoter
MYD88: myeloid differentiation primary response gene 88
Myf5: myogenic factor 5
MyHC: cardiac myosin heavy chain
MYOD1: myogenic differentiation gene or protein 1
MYTH11: a smooth muscle myosin
Mx-1: myxovirus resistance protein-1
NaCl: sodium chloride
NAD: nicotinamide adenine dinucleotide
NADH: the reduced form of nicotinamide adenine dinucleotide
NADPH oxidase: nicotinamide adenine dinucleotide phosphate oxidase
NAP-2: neutrophil-activating protein 2
NASA: National Aeronautics and Space Administration (USA)
N-cadherin: neuron cadherin (cadherin = calcium-dependent adhesion)
N-CAM: neural adhesion molecule
NCOA4: nuclear receptor coactivator 4
NDGA: dicatechol nordihydroguaiaretic acid
NDRG1: N-myc downstream-regulated gene 1
NEP: neutral endopeptidase
NeuN: neuronal nuclear antigen
NEV: neutrophil extracellular vesicle
NF1B: nuclear factor 1 B-type
NFAT: nuclear factor of activated T cells
NFATC1: nuclear factor of activated T cells, cytoplasmic 1
NF200: 200 kDa neurofilament protein
NFκB: nuclear factor kappa light chain-enhancer of activated B cells
NG2: neural/glial antigen 2
NGF: nerve growth factor

NHEJ: nonhomologous end-joining
NIH: National Institutes of Health (USA)
NIHSS: National Institutes of Health Stroke Scale or NIH Stroke Scale
NIPAL3: NIPA-like domain containing 3
NRIP1: nuclear receptor-interacting protein 1
NK: natural killer
NK4: N-terminal hairpin and subsequent four kringle domains of hepatocyte growth factor
NKG2D: natural killer cell lectin-like receptor subfamily K, member 1
NKX3.2: NK3 homeobox 2
NLR: Nod-like receptor
NLRP3: NOD-like receptor family, pyrin domain containing 3
nNOS: neuronal NO synthase
NO: nitrous oxide
NOD: nonobese diabetic (mice)
NOS: nitrous oxide synthetase
Notch 1: Notch homolog 1, translocation-associated (*Drosophila*)
NOV: nephroblastoma overexpressed (also known as CCN3)
NP: nanoparticle
NPC: neural progenitor cell
NRG1-β1: neuroregulin-β1
NRP1: neuropilin-1
NSAID: nonsteroidal anti-inflammatory drug
NSC: neural stem cell
NSC: neural stromal cell
NSG: NOD SCID Gamma (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ – a strain of inbred laboratory mice, among the most immunodeficient described to date. NSG mice lack mature T cells, B cells, and natural killer cells. NSG mice are also deficient in multiple cytokine signaling pathways and they have many defects of innate immunity)
NS-MSC: neurosphere-like cellular aggregates
NSPC: neural stem/precursor cells
NSS: neurologic severity score
NT: neurotrophin
NTA: nanoparticle tracking analyses
NTT: Nordon's Tracking Tool
NVU: neurovascular unit
NYHA: New York Heart Association
$O_2$: oxygen
OA: osteoarthritis
OB: obliterative bronchiolitis
OB-cadherin: osteoblast cadherin (cadherin = calcium-dependent adhesion)
OC: osteocalcin
OCN: osteocalcin
OCIF: osteoclastogenesis inhibitory factor (now known as osteoprotegerin)
OCT-4: octamer-binding transcription factor
OEG: olfactory glia
OGD: oxygen-glucose deprived
OH: hydroxyl
OI: osteogenesis imperfecta
OKT3: monoclonal antibody against CD3 (a pan T cell surface antigen)
OMG: oligodendrocyte-myelin glycoprotein
OP-1: osteogenic protein-1
OPG: osteoprotegerin
OPN: osteopontin
OPPG: osteoporosis-pseudoglioma syndrome
ORF: open reading frame
ORP8: oxysterol-binding protein-related protein 8
OSE2: osteoblast-specific cis-acting element 2
OSR1/2: odd-skipped related 1/2
OSX: Osterix (an osteoblast-specific transcription factor essential for osteoblast differentiation and bone formation)
OVA: ovalbumin
OVX: ovariectomy
OXi4503: combretastatin A1 di-phosphate/CA1P (a dual-mechanism vascular disrupting agent)
OXPHOS: oxidative phosphorylation
P: passage (number)
P1NP: propeptide of type 1 procollagen
p53 (or TP53): tumor protein 53 was the name given in 1979 describing a protein mass of 53 kDa to a gene that encoded a protein that acted as a tumor suppressor gene
p63: transformation-related protein 63
PA: polyamide
PACAP: pituitary adenylate cyclase-activating polypeptide

PAD: peripheral arterial disease
PAF: platelet-activating factor
PA/G: polyamide gelatin
PAH: pulmonary artery hypertension
PAI-1: plasminogen activator inhibitor 1
PAK: p21-activating kinases
pAkt: phospho-Akt (for Akt see under A above)
PAMPs: pathogen-associated molecular patterns
PANTHER: protein analysis through evolutionary relationships
PAOD: peripheral arterial occlusive disease
PAR: poly-(ADP) ribose
PARK2 gene: mutations in this gene are known to cause a familial form of Parkinson's disease[6]
PARP: poly-(ADP) ribose polymerase
PAX: paired box
PBF1: proteasome beta subunit F1
PBMC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
PC12: a cell line derived from a pheochromocytoma of the adrenal medulla
PCI: percutaneous intervention
PCLA: poly-ε-caprolactone and lactic acid
PCNA: proliferating cell nuclear antigen
PCP: planar cell polarity
PCR: polymerase chain reaction
PCR2: poly comb repressor complex 2
PD: Parkinson's disease
PD: population doubling
PD-1: programmed cell death protein 1 (also known as CD279)
PDAI: perianal disease activity index
PDCD6IP: programmed cell death 6-interacting protein (also known as Alix)
PDEF: pigment epithelium-derived factor (also known as serpin F1)
PDGF: platelet-derived growth factor
PDGFA: platelet-derived growth factor-α (also known as PDGF-1)
PDGFB: platelet-derived growth factor subunit B
PDGFBB: platelet-derived growth factor BB (homodimer)
PDGFR: platelet-derived growth factor receptor
PDK1: phosphoinositide-dependent kinase-1
PDK1: pyruvate dehydrogenase lipoamide kinase isozyme 1
PD-L1: programmed death ligand 1
PDLIM4: PDZ and LIM domain 4
PDMS: polydimethylsiloxane
PDX-1: pancreatic duodenal homeobox-1
PDZ: PDZ is an acronym combining the first letters of three proteins – post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1), which were the first proteins discovered to share the PDZ domain
PECAM-1: platelet endothelium adhesion molecule-1
PECAM-1: platelet/endothelial cell adhesion molecule-1 (CD31)
PEG: polyethylene glycol
PEMF: pulsed electromagnetic field
PET: positron emission tomography
PEV: platelet extracellular vesicle
PFD: pelvic floor disorder
PFKFB3: phosphofructokinase-2/fructose-2,6-bisphosphatase 3
PG: proteoglycans
PGE: prostaglandin E
PGF: placental-derived growth factor
PGK: phosphoglycerate kinase
PGM: phosphoglycerate mutase
PGN: peptidoglycan
PGP9.5: protein gene product 9.5
PHA: phytohemagglutinin
PHD: prolyl hydroxylase domain-containing protein
PHEMA: poly(2-hydroxyethyl methacrylate)
Phf2: plant homeodomain zinc finger-like 2
PI: protease inhibitor
PIC/S: Pharmaceutical Inspection Convention (PIC) and Pharmaceutical Inspection Co-operation Scheme
PI3K: phosphatidylinositide 3-kinase
PIG: proliferation inducing gene
PIK: phosphatidylinositide 3-kinases
PIK3CD: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit delta
PINK1: PTEN-induced putative kinase 1
PK: pyruvate kinase

PKA: protein kinase A (A family of enzymes whose activity is dependent on cellular levels of cyclic AMP (cAMP). PKA is also known as cAMP-dependent protein kinase. Protein kinase A has several functions in the cell, including regulation of glycogen, sugar and lipid metabolism)

PKB: protein kinase B

PKm2: pyruvate kinase isozyme M2

PKP: penetrating keratoplasty

PL: platelet lysate

PLA: poly(L-lactic acid) scaffold

PLA: polylactic acid

PLAU: urokinase-type plasminogen activator (encoded by the PLAU gene)

PLAUR: urokinase-type plasminogen activator receptor

PLGA: poly(lactic-co-glycolic acid)

PLGA: polyglycolic acid

PLGF: placental growth factor

PMDA: Pharmaceutical and Medical Devices Agency

pMSC: placental mesenchymal stromal cell

PNCA: proliferating cell nuclear antigen

PNS: peripheral nervous system

PODXL: podocalyxin-like protein

POLR2E polymerase (RNA) II (DNA directed) polypeptide E gene

polyI:C: polyinosinic:polycytidylic acid

POP: pelvic organ prolapse

POU3F1: POU domain, class 3, transcription factor 1

PP: polypropylene

PPAR: peroxisome proliferator-activated receptor

pQCT: peripheral quantitative computed tomography

PRAK: p38-regulated/activated kinase

Pre-RC: pre-replication complex

PRDM16: PR domain containing 16

pre-miRNA: precursor miRNA

PRG4: proteoglycan 4

PRGF: platelet-released growth factors

pri-miRNA: primary miRNA

PRMT: protein arginine methyltransferase

PRP: platelet-rich plasma

PRR: pattern recognition receptor

PRRX1: paired related homeobox 1

PSGL-1: P-selectin glycoprotein ligand-1

PTEN: phosphatase and tensin homolog

PTH: parathyroid hormone

PTHLH: parathyroid hormone-like hormone

PTHR1: parathyroid hormone receptor

PTHrP: parathyroid hormone-related protein

PTIP: PAX transactivation activation domain-interacting protein

PTN: pleiotrophin – also known as heparin-binding brain mitogen (HBBM), heparin-binding growth factor 8 (HBGF-8), neurite growth-promoting factor 1 (NEGF1), heparin affinity regulatory peptide (HARP), or heparin binding growth associated molecule (HB-GAM)

PTM: posttranslational histone modification

PTX3: pentraxin-related protein

PU 1: nuclear protein that binds to a purine-rich sequence known as the PU-box found near the promoters of target genes

PU 1 AS: PU 1 antisense

PUCAI: pediatric ulcerative colitis activity index

PUVA: psoralen and ultraviolet-A light

pVHL: product of the von Hippel–Lindau (tumor suppressor)

PYCARD/ASC: apoptosis-associated speck-like protein

QA: quality assurance

QC: quality control

QD: quantum dot

qRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction

RA1: (putative) drug resistance ATPase-1

Rab: Ras-related in brain (the Rab family of proteins is a member of the Ras superfamily of monomeric G proteins)

RAC1: Ras-related C3 botulinum toxin substrate 1

RAD51: a DNA repair protein

RALA: v-ral simian leukemia viral oncogene homolog A

RANK: receptor activator of nuclear factor κB

RANKL: RANK ligand
RANTES: regulation on activation, normal T cell expressed and secreted (also known as CCL5)
RAR: retinoic acid receptor
RAS: a family of proteins involved in cellular signal transduction first discovered in a rat sarcoma line
Rb: retinoblastoma
RBP2: retinoblastoma binding protein 2
RBP-J: recombination signal binding protein-J
R-cadherin: retinal cadherin
RCAS: the RCAS family of plasmids were originally derived from a molecular clone of the SR-A strain of Rous sarcoma virus
rCE: rabbit carboxylesterase enzyme
RECA: rat endothelial cell antigen
RCT: randomized controlled trial
Re-Seq: reference sequence promoter arrays
REV: red blood cell extracellular vesicle
REX1: reduced expression gene 1
RFP: red fluorescent protein
RGD: the tripeptide arginine, glycine, aspartic acid (Arg–Gly–Asp). The sequence is a common element in cellular recognition
RGS5: regulator of G-protein signaling
RHO: Ras homolog gene family
rHo: Spearman's rank-order correlation coefficient
RIF1: Rap1-interacting factor
RIG: retinoic acid inducible
RISC: RNA-induced silencing complex
RISK: reperfusion injury salvage kinases
RNA: ribonucleic acid
RNAi: RNA interference
RNS: reactive nitrogen species
ROBO4: roundabout homolog 4, also known as magic roundabout (gene, protein)
ROCK: Rho-associated protein kinase, RhoA-Rho kinase
ROI: region of- interest
RORγ: retinoic acid-related orphan receptor gamma
ROS: reactive oxygen species
RPA: replication protein A
RPLC: reversed-phase liquid chromatography
RR: relative risk
rRNA: ribosomal RNA
RNF: E3 ubiquitin ligase RING finger protein
RSK: ribosomal 6s kinase
R-SMAD: receptor-regulated SMAD
RT-PCR: reverse transcriptase polymerase chain reaction
RUNX2: runt-related transcription factor 2
RUUO: reversal of the unilateral ureteric obstruction
RVG: rotavirus genus or rotavirus G
RVH: right ventricular hypertrophy
RWV: rotary wall vessel
RXFP: relaxin family peptide receptor
S: (DNA) synthesis (phase of the cell cycle)
S: Svedberg sedimentation coefficient unit[7]
S100-A9: a protein that in humans is encoded by the S100A9 gene (also known as migration inhibitory factor-related protein 14 (MRP-14) or calgranulin-B)
SAA: serum amyloid A
SABA: short-acting beta agonist
SAG: a small-molecule agonist of the Sonic hedgehog-Smoothened (Shh-Smo) signaling pathway
SAMP6: senescence-accelerated mouse strain
SAPK: stress-activated protein kinase
Sca-1: stem cell antigen
SCC: squamous cell carcinoma
SCF: stem cell factor (c-kit ligand)
SCGB1A1: secretoglobin family 1A member 1 (also known as uteroglobin)
SCI: spinal cord injury
SCID: severe combined immunodeficiency
sCMOS: scientific complementary metal-oxide-semiconductor
SCX: strong cation exchange chromatography
SDF-1: stromal cell-derived factor-1 (also known as CXCL12)
SDS-PAGE: polyacrylamide gel containing sodium dodecyl sulfate

SEB: staphylococcal enterotoxin B
SEM: scanning electron microscopy
Sema3a: semaphorin3A
SEP: sensory evoked potentials
SERCA: sarco/endoplasmic reticulum $Ca^{2+}$-ATPase, or SR $Ca^{2+}$-ATPase
SERPIN: serine proteinase inhibitor (superfamily)
SERPINE1: serpin peptidase inhibitor, clade E
SETDB1: set domain bifurcated 1
SFM: serum-free medium
SFRP: secreted frizzled-related protein
SFTPC: surfactant protein C
SH2: Src homology 2-like (domains)
SHH: Sonic hedgehog (Hedgehog was first discovered in the fruit fly by researchers who thought the spiky protein looked like a hedgehog. Sonic was named as such by the Harvard researcher (Cliff Tabin) because two previous gene family members had been named Indian and desert hedgehog based on the way fruit flies looked when these genes were mutated. His children were playing a lot of Sonic the Hedgehog video games at the time he discovered his gene (circa 1991) so he went with that name for the third member of this protein family)
SHH-SMO: Sonic hedgehog-Smoothened
sHLA-G: soluble human leukocyte antigen
SHP: small heterodimer partner, also known as NR0B2 (nuclear receptor subfamily 0, group B, member 2). SHP is a member of the nuclear receptor family of intracellular transcription factors. SHP is unusual for a nuclear receptor in that it lacks a DNA binding domain. Therefore, technically it is neither a transcription factor nor a nuclear receptor, but nevertheless it is still classified as such due to relatively high sequence homology with other nuclear receptor family members
shRNA: short hairpin RNA or small hairpin RNA
SHR-SP: spontaneous hypertension rat stroke-prone
SILAC: stable isotope labeling by amino acids in cell culture
SIM2: single-minded homolog 2
siRNA: small interfering RNA
SIRS: systemic inflammatory response syndrome
SIS: small intestinal submucosa
SLAM: signaling lymphocyte activation molecule
SLC: secondary lymphoid-tissue chemokine
SLE: systemic lupus erythematosus
SLeX: sialyl Lewis X (cell adhesion molecule)
SLRP: small leucine-rich repeat proteoglycan (also known as biglycan)
SMA: smooth muscle actin
SMAD: SMAD proteins are homologs of both the *Drosophila* protein, mothers against decapentaplegic (MAD), and the *Caenorhabditis elegans* protein SMA (from gene SMA for small body size)
SMB: somatomedin B
SMC: smooth muscle cell
SM-MHC: smooth muscle myosin heavy chain
SMRT: silencing mediator of retinoid and thyroid hormone receptors
SREBP-1: sterol regulatory element binding protein 1
SN: substantia nigra
SNARE: N-ethylmaleimide-sensitive factor activating protein receptor
SOCS or COCS: suppressor of cytokine signaling
SOST: sclerostin
SOSTdc-1: sclerostin-domain containing protein-1
SOT: solid organ transplantaion
SOX: Sry-related high mobility group (HMG) box
SP: sulfophenyl
SPARC: secreted protein acidic and rich in cysteine (also known as osteonectin)
SPECT: single-photon emission computed tomography
SPEDF: SAM pointed domain containing ETS transcription factor
SPIO: superparamagnetic iron oxide

SPP 1: secreted phosphoprotein 1 (also known as osteopontin)
src: Schmidt–Ruppin A-2 viral oncogene homolog
SRF: serum response factor
SRM: selected reaction monitoring
SRY: sex-determining region Y-box 9 (also known as SOX9)
SSEA: stage-specific embryonic antigen
SSB: single-strand break
SSEP: somatosensory evoked potential
SSI: STAT-induced STAT inhibitor, also known as suppressor of cytokine signaling (SOCS)
STAT: signal transducer and activator of transcription
STC-1: stanniocalcin-1
STEMI: ST-segment elevation acute myocardial infarction
sTNFR1: soluble tumor necrosis factor receptor 1
sTRAIL: soluble variant of tumor necrosis factor-related apoptosis-inducing ligand
Stro-1: stromal precursor antigen-1
SUMO-1: small ubiquitin-related modifier 1 gene
SUSD2: sushi domain containing 2
SV40: simian vacuolating virus 40 or simian virus 40
SVEC: SVEC4-10 is a murine endothelial cell line derived by SV40 transformation of endothelial cells from axillary lymph node vessels
SVF: stromal vascular cell fraction (of fat, containing preadipocytes, fibroblasts, vascular endothelial cells, and a variety of immune cells, such as adipose tissue macrophages)
SVZ: subventricular zone
SWI/SNF: SWItch/sucrose nonfermentable
SY-MSCs: synovial membrane MSCs
T1DM-MCAO: type 1 diabetes mellitus rats which have undergone a middle cerebral artery occlusion resulting an ischemic stroke
TAM: tumor-associated macrophage
TA-MSC: tumor-associated MSC
TAO: thromboangitis obliterans
TAZ: transcriptional co-activator with PDZ-binding motif
TB4: thymosin-β4
T-bet: T-box transcription factor TBX21 is a protein that in humans is encoded by the TBX21 gene
TβR1: transforming growth factor beta type I receptor
TBX: T-box
Tc: cytotoxic T cells
TCF: transcription factor
TCF: T cell factor
TCF4: transcription factor 7-like 2 (sometimes referred to as immunoglobulin transcription factor 2TCF4)
TCF7: transcription factor 7-like 2 (T-cell specific, HMG-box)
TCF7L2: transcription factor 7-like 2 (T-cell specific, HMG-box) (also known as TCF7)
TCP: tricalcium phosphate
TcPO2: transcutaneous partial oxygen pressure
TCR: T cell receptor (for antigen)
tdTomato: tandem dimeric derivative of DsRed
TDZD: thiadiazolidinone
TE: tissue engineering
TEC: thymic epithelial cell
TECK: thymus-expressed chemokine
TEM: transmission electron microscopy
TERT: telomerase reverse transcriptase
TET1–3: ten–eleven translocation 1–3
TF: tissue factor (also known as platelet tissue factor, factor III, thromboplastin, and CD142)
TF: transcription factor
TFEV: tissue factor expressing extracellular vesicle
TGA: Therapeutic Goods Authority (of Australia)
TGF-β: transforming growth factor-β
tgHD: transgenic Huntington disease (rat)
TGP: thermoreversible gelation polymer
Th: helper T cells
THBD: thrombomodulin
Thy1: thymocyte antigen 1 (CD90)
TIA: transient ischemic attack

TIE: tyrosine kinase with immunoglobulin-like and EGF-like domains
TIFF: tagged image file format
TIL: tumor-infiltrating lymphocyte
TIMP: tissue inhibitor of metalloproteinases
Tip60: tat interactive protein-60
TIR: toll/interleukin-1 receptor homology domain
TIRF total internal reflection microscopy
TK: thymidine kinase
TLR: toll-like receptor
TM4SF: transmembrane 4 superfamily
tMVECS: tumor microvascular endothelial cells
TNAP: tissue nonspecific alkaline phosphatase
TNBS: trinitrobenzene sulfonate
TNFAIP6: tumor necrosis factor-α-induced protein 2
TNFR: tumor necrosis factor receptor
TNF-α: tumor necrosis factor-α
TNTs: tunneling nanotubes
TOLL: Toll-like receptors (TLRs) received their name from their similarity to the protein coded by the toll gene identified in *Drosophila* in 1985 by Christiane Nüsslein-Volhard. The researchers were so surprised that they spontaneously shouted out in German "Das ist ja toll!," which translates as "That's great!"
tPA: tissue plasminogen activator
TPM: tropomyosin
Tr1: type 1 Tregs
TRA-1-60: tumor rejection antigen-1-60
TRA-1-81: tumor rejection antigen-1-81
TRACP5b: tartrate-resistant acid phosphatase 5b
TRADD: tumor necrosis factor receptor type 1-associated death domain protein
TRAF: TNF receptor-associated factor
TRAIL: tumor necrosis factor-related apoptosis-inducing ligand (also known as CD253 and tumor necrosis factor (ligand) superfamily, member 10 (TNFSF10))
TRANCE: TNF-related activation-induced cytokine (now known as RANK)
TRAP: tartrate-resistant acid phosphatase
TREC: T cell receptor excision circle
Treg: regulatory T cell
TRIF: TIR domain-containing molecule
TRIP-10: thyroid receptor-interacting protein 10
TrcK: tropomyosin receptor kinase C (also known as NT-3 growth factor receptor or neurotrophic tyrosine kinase receptor type 3)
tRNA: transfer RNA
TRPC6: transient receptor potential canonical 6
Tsg101: tumor susceptibility gene 101
TSG-6: tumor necrosis factor α-induced protein-6
TSH: thyroid-stimulating hormone
TSLP: thymic stromal lymphopoietin
TSP: thrombospondin
TSS: transcription start site
TTT: Timm's Tracking Tool
TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling
tv-a: a gene encoding the retrovirus receptor
TWIST 1: Twist family bHLH transcription factor 1
U0126: a highly selective inhibitor of both MEK1 and MEK2, a type of MAPK/ERK kinase
Ub: ubiquitin
UC: ulcerative colitis
UC: umbilical cord
UCB: umbilical cord blood
UCLA: University of California, Los Angeles
UCMSCs: umbilical-cord-derived mesenchymal stromal cells
ULA: ultralow attachment (plates)
uPAR: soluble urokinase-type plasminogen activator receptor (CD87)
USPIO: ultra-small superparamagnetic iron oxide
USSC: unrestricted somatic stem cell
UTR: untranslated region (of DNA)
UTX: ubiquitously transcribed tetra-tricopeptide repeat X
UUO: unilateral ureteric obstruction
UV: ultraviolet
uVA: urokinase-type plasminogen activator

VAS: visual analog scale
VCAM-1: vascular cell adhesion molecule 1
VCAN: versican
VDJ: variable (V), joining (J) and diversity (D) (gene segments of B cells)
VE-cadherin: vascular endothelial cadherin
VEGF: vascular endothelial growth factor
VEGFR: vascular endothelial growth factor receptor (also known as KDR)
VHL: von Hippel–Lindau
VLA-4: very late activation antigen-4 (CD49d)
VLA-5: very late activation antigen-5 (CD49e)
VPA: valproic acid
VSEL: very small embryonic-like (cells)
VSMC: (blood) vessel-derived vascular smooth muscle cell
VSV-G: vesicular stomatitis virus-G
VTCN1: V-set domain-containing T-cell activation inhibitor 1
vWF: von Willebrand factor
WHO: World Health Organization
WIF: wnt inhibitory factor
WJ-MSC: Wharton's jelly mesenchymal stromal cell
Wnt: wingless-related integration site
WT: wild type
WWP2: WW domain containing E3 ubiquitin protein ligase 2
X4-L4: a complex consisting of X-ray repair cross-complementing protein 4 (XRCC4), XRCC4-like factor (XLF), and DNA ligase IV
xeno-free: free from any contamination by components of non-human species
X-Gal: P3-D-galactoside (commonly used to test for the presence of β-galactosidase)
XLF: XRCC4-like factor
XRCC4: X-ray repair cross-complementing protein 4
XYLT: xylosyltransferases
YAP: Yes-associated protein
YFP: yellow fluorescent protein
YY1: transcriptional repressor protein yin and yang 1
ZAP-70: zeta-chain-associated protein kinase 70
ZIMZI: zinc finger myc-interacting protein-1
ZO-1: zonula occludens-1
Z-VAD: *Z*-Val-Ala-DL-Asp-FMK (a caspase family inhibitor and cell permeable apoptotic protease)

## Historical notes

1. Alzheimer: Alzheimer's disease was first described by, and later named after, the German psychiatrist and pathologist Alois Alzheimer in 1906.
2. Dulbecco: Dulbecco refers to Renato Dulbecco. who, with David Baltimore and Howard Temin, won the Nobel Prize in Physiology or Medicine in 1975.
3. Eagle: Eagle refers to Harry Eagle. who developed the medium for the culture of HeLa cells and mouse fibroblasts.
4. Huntington: In 1872, at the age of 22, George Huntington described the disorder in his first paper, "On Chorea".
5. Iscove: In 1976, Guilbert and Iscove demonstrated that precursor cells of erythrocytes and macrophages could be cultured in a reduced-serum medium supplemented with albumin, transferrin, lecithin, and selenium. Iscove's medium is a variation of Dulbecco's modified Eagle's Medium (DMEM) containing selenium, additional amino acids and vitamins, sodium pyruvate, HEPES buffer, and potassium nitrate instead of ferric nitrates.
6. Parkinson: The disease is named after the English doctor James Parkinson, who published the first detailed description in "An Essay on the Shaking Palsy" in 1817.
7. Svedberg: the S unit was named after the Swedish chemist Theodor Svedberg (1884–1971), winner of the 1926 Nobel Prize in Chemistry for his work on disperse systems, colloids, and his invention of the ultracentrifuge.

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