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Preface

"Every animal appears as a sum of vital units, each of which bears in itself the complete characteristics of life," says Rudolf Virchow in 1858. More than a century and a half has passed since these words were uttered, and science has dissected the cell, so to speak, down to its most minute components. The progress made in medicine is simultaneously remarkable and severely inadequate. We expect that continued progress in fields like microscopy, multi-omics, CRISPR/Cas9 gene editing, organoids, organ-on-chip systems, epigenetic engineering, machine learning, and other technologies will deepen our understanding of the molecular processes involved in reprogramming somatic cells into pluripotent cells and differentiating induced pluripotent stem cells (iPSCs) into specific cell types. However, only those in the field commonly recognize key discoveries in cell biology.

Subsequent to the discovery and isolation of stem cells from human tissues, the generation of iPSCs stands as one of the most groundbreaking advancements. Despite numerous groundbreaking discoveries in biology published in high-impact journals, many have had little or no discernible impact on society, even temporarily. For instance, research has demonstrated that plant microRNAs can migrate into a host's blood and tissues through the consumption of food. This suggests that "consuming material" may also involve "consuming information," supporting the concept of epigenetic regulation. Furthermore, nonlinear dynamics are shedding light on stem cell fates, highlighting the synergy between mathematics and cell biology. Despite these and other significant discoveries, their broader societal implications have not been fully recognized.

Media coverage of stem cell research and development is proliferating, with hardly a day passing without news headlines on the topic. Unfortunately, this intense media attention often obscures the distinction between fact and fiction. The creation of bio-organs and miraculous cures for cancer and other critical diseases are frequently featured in daily news. Similarly, many dental practitioners envision the creation of bio-teeth with the aid of stem cells. However, the primary focus of this book is not whether dental stem cells can generate a tooth but rather the significant challenges that remain within the field of dental stem cell research.

The exorbitant costs associated with dental education often compel dental professionals to seek high-paying positions immediately upon graduation. This financial pressure is a significant factor contributing to the scarcity of tooth-related discoveries, as the number of dental surgeons with PhD qualifications is declining, particularly in developing countries. Moreover, compared to the late eighteenth and early nineteenth centuries, basic dental sciences have lost popularity worldwide.

The tooth and its surrounding oral environment constitute one of the most intricate and adaptive systems within the human body. Teeth must withstand constant bacterial assault from the oral cavity, tolerate extreme temperature fluctuations, and endure substantial forces during mastication. The highly specialized mesenchymal tissue, dental pulp, is encased within the rigid, mineralized dentin tissue. This pulp-dentin complex forms an integrated organ characterized by unique interactions between mineralized and soft tissues, with a remarkable regenerative capacity.

Despite its small size, the dentin-pulp organ offers a vast scope for research in fields such as development, differentiation, regeneration, immunoregulation/immunomodulation, epigenetics, genomics, proteomics, and others. Routinely discarded after extraction, permanent and exfoliated primary teeth should be considered for these studies as they are ethically and politically uncontroversial human tissues. Moreover, they can be obtained noninvasively. Dental pulp cells readily provide suitable material for studying various aspects of cell biology, including clinical applications.

I am grateful for the opportunity to have worked with so many brilliant minds throughout my career. And last but not least, thanks to Muammer Saglam for giving me an unquenchable flambeau and for his unconditional love that guided me to find my unique way.

I am compelled to express my sorrow at witnessing the progressive estrangement of my nation, distinguished by its multifaceted uniqueness, from the domains of scientific inquiry and artistic expression. Concurrently, I am filled with admiration for the relentless pursuit and unwavering faith in the legacy of Atatürk's Turkey exhibited by my country's scientific community, despite facing considerable challenges and limited support.

Bilecik, Türkiye Sibel Yildirim, DDS, PhD, PhD

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About the Author

Sibel Yildirim, DDS, PhD, PhD is professor at the Faculty of Dentistry, Department of Pediatric Dentistry, Bilecik Seyh Edebali University, Bilecik, Turkey. She has been a lecturer and practicing dentistry at the university for years. Her colleagues and she have established the first multi-disciplinary, dental/oral research center in Faculty of Dentistry, Selcuk University, Turkey. She has authored many papers in international journals and has completed several research projects. Along with her pediatric dentistry PhD, she has also her second PhD on histology and embryology. Her research interests include many aspects of dental pulp and induced pluripotent stem cells, deciduous tooth resorption, the viral ethiopathogenesis of pulpal/periapical diseases of deciduous teeth, and vital regenerative pulp therapies using recombinant human proteins or gene therapy. Accordingly, she has led some experiments on deciduous tooth pulp tissue, and her team obtained important results showing high regulatory capacity of dental pulp cells on the events of deciduous tooth resorption or retention.

She had a change to study with the indisputable leaders in the dental engineering field in Japan, Switzerland, and the United States. She has become an expert for stem cells from the pulp tissue of deciduous and permanent teeth. In addition to her dental tissue engineering studies, she has pursued re-programming experiments. She could observe that stem cells that have dental origin displayed strong potential for reprogramming. She believes comprehensively that isolating epithelial and mesenchymal stem cells from deciduous teeth and reprogramming them will point toward novel venues for in situ restoration of dental tissue repair. Currently her ongoing projects are focused on epigenetic regulations in dental pulp tissue.

As a pediatric dentist, histologist, and embryologist, after long years of experience in academic fields in different continents, she has found herself that she became a student again who is keen to reach beyond the traditional boundaries of biology. She totally convinced that complexity is the only field that helps to achieve her goals; moving one step closer to understanding life.

Abbreviations

NC	Neural crest cells
FGF	Fibroblast growth factor
BMP	Bone morphogenetic protein
DPSC	Dental pulp stem cells
SHED	Stem cells from human exfoliated deciduous teeth
ED	Embryonic day
SHH	Sonic hedgehog
WNT	Wingless integrated
PAX	Paired box gene
RUNX	Runt-related transcription factor
IDE	Inner dental epithelium
ODE	Outer dental epithelium
BM	Basal membrane
BSP	Bone sialoprotein
DSP	Dentin sialoprotein
DSPC	Dental pulp derived mesenchymal stromal cells
DPP	Dentin phosphoprotein
DSPP	Dentin sialophosphoprotein
DMP	Dentin matrix protein
OPN	Osteopontin
SIBLING	Small integrin-binding ligand, N-linked glycoproteins
MEPE	Matrix extracellular phosphoglycoprotein
HLA	Human leukocyte antigen
MHC	Major histocompatibility complex
CD	Cluster of differentiation
CGRP	Calcitonin gene-related peptide
H&E	Hematoxylin-eosin staining
TNF	Tumor necrosis factor
TGFβ	Transforming growth factor β
MAPK	Mitogen-activated protein kinases
3HTdR	Tritiated thymidine

MSC	Mesenchymal stem cells
USCS	Umblical cord stromal cells
NS	Nucleostemin
TRAP	Tartrate resistance acid phosphatase
DGJ	Dento-gingival junction
RANKL	Receptor activator nuclear factor kappa B ligand
OPG	Osteoprotegerin
IDPSC	Immature dental pulp stem cells
FACS	Fluorescence activated cell sorting
VEGF	Vascular endothelial growth factor
MEM	Eagle's minimum essential medium
DMEM	Dulbecco's modified essential medium
F12	Ham's nutrient mixture
BME	Eagle's basal medium
RPMI	Roswell Park Memorial Institute medium
IGF	Insulin-like growth factor
EGF	Epidermal growth factor
FBS	Fetal bovine serum
FCS	Fetal calf serum
DMEM-KO	Dulbecco's modified Eagle's medium knock out
P	Passage
SSEA	Stage-specific embryonic antigen
Oct4	Octamer-binding transcription factor 4
Sox2	Sex determining region Y-box 2
TRA-1	Tumor recognizing antigen 1
Msx1	Msh homeobox 1
IBMX	3-Isobutyl-1-methylxanthine, like other methylated xanthine derivatives
iPSC	Induced pluripotent stem cells
LRP	Low-density lipoprotein receptor-related protein
IDO	Indoleamine 2,3-dioxygenase
PGE	Prostaglandin E
NO	Nitric oxide
IFN	Interferon
IL	Interleukin

Chapter 1
Mesenchymal Stem Cells

Cohnheim first proposed in 1867 that non-hematopoietic stem cells within the bone marrow were involved in wound healing. Subsequently, hematopoietic stem cells were identified in bone marrow stroma in 1960. The term "mesenchymal stem cells" was coined in 1991 to describe non-hematopoietic stem cells with stem cell properties found in the bone marrow stroma. Human mesenchymal stem cells were first isolated in 1999. Since these seminal discoveries, mesenchymal stem cells have been derived from a variety of tissues, including human dental pulp in 2000.

For the past 24 years, the dental community has anticipated the clinical application of stem cell therapies. Despite a growing body of stem cell research, particularly focusing on the readily accessible dental pulp, the translation of these findings into clinical practice remains elusive. This discrepancy between the volume of research and the paucity of clinical treatments necessitates a critical examination of the factors hindering the advancement of stem cell-based dental therapies.

This book aims to provide dental scholars and practitioners with a comprehensive overview of stem cell biology, challenging them to consider multiple perspectives on this complex topic. As a pediatric dentist with a background in cell biology, I believe this interdisciplinary approach is essential. To address the complexities of stem cell research and its clinical implications, we will begin by examining the historical development of the field. This historical perspective will illuminate the evolution of our understanding of stem cells, from their conceptual origins to their contemporary characterization. Subsequently, we will delve into the fundamental biological properties of stem cells, exploring the theoretical framework that underpins their potential applications in regenerative medicine. A critical analysis of the discrepancies between the idealized properties of stem cells and the characteristics of those isolated in the laboratory will be presented. The initial wave of enthusiasm surrounding stem cell-based therapies, often accompanied by unregulated practices, serves as a cautionary tale. By scrutinizing these early experiences, we can assess whether laboratory-derived stem cells possess the intrinsic properties necessary for safe and efficacious clinical translation.

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Following an overview of stem cells, this book will expand upon its predecessor by incorporating updated chapters that address recent advancements in dental pulp-derived mesenchymal stromal cell researches. The primary objective is to stimulate innovative therapeutic approaches by highlighting the ethical and abundant nature of dental tissues as a source of human stem cells. This resource is uniquely positioned to facilitate extensive investigations into underlying biological mechanisms. It is the author's aspiration that this work will catalyze the development of novel treatments and perspectives within the field.

1.1 Multipotent Mesenchymal Stromal Cells: A Historical Perspective

The discovery story of stem cells stretches back to the late nineteenth century. Scientists like Cohnheim and Maximov laid down the basics of the idea underlying stem cells in the nineteenth and twentieth centuries. According to Kfoury and Scadden (Kfoury & Scadden, 2015), Cohnheim observed the presence of non-hematopoietic stem cells in year 1867 in the host's bone marrow that contributed to wound healing through fibroblast generation and collagen deposition. Maximov later, in 1924, identified a significant interaction of the newly developing blood components with the mesoderm during embryonic development. These early observations suggested that the stromal cell reservoir of the bone marrow takes part in both natural healing and hematopoiesis (Parekkadan & Milwid, 2010). In 1968, Tavassoli and Crosby (1968) reported the presence in a bone of a further and different cell population that showed both proliferative and osteoblastic potentials. Such observations were suggested to indicate the presence of a non-hematopoietic stem cell population capable of differentiating into the bone (Tavassoli & Crosby, 1968).

Hematopoietic stem cells (HSCs) were discovered in the 1960s by two Canadian investigators, Drs. James Till and Ernest McCulloch (McCulloch & Till, 1960). They conducted experiments in the early 1960s to transplant bone marrow cells into irradiated mice. In their pioneering work, they identified a rare population of cells within the bone marrow that could give rise to all types of blood cells. They referred to these as "stem cells" because they can effectively develop and differentiate into all types of blood cells.

The story of mesenchymal stem cells, specifically regarding their early observation and initial isolation, begins with the pioneering work of Alexander Friedenstein. His observations of the late 1960s were a clue that there was a distinct stem cell population within the bone marrow differentiating into the bone (Fridenshteĭn et al., 1968). During the 1970s, he isolated and cultured the cells from guinea pig bone marrow that would later be named "osteogenic stem cells" due to their osteoblastic formational potential by others (Friedenstein et al., 1970). Friedenstein et al. (1974) further characterized the cells and demonstrated that they could not only

differentiate into the bone but were also capable of forming the cartilage and fat tissue (Friedenstein et al., 1974). Because of these cells' potential to form osteoblasts, they were termed osteogenic stem cells (Fridenshteĭn et al., 1968; Friedenstein et al., 1970).

Therefore, Friedenstein was a pioneer in isolating adherent, fibroblast-like cells from the bone marrow, subsequently termed colony-forming unit-fibroblasts (CFU-Fs). These cells exhibited a remarkable proliferative capacity and the ability to differentiate into various non-hematopoietic cell types, ultimately leading to the in vivo formation of a bone tissue. Notably, Friedenstein and colleagues were the first to report the in vitro differentiation of these cells into not only osteocytes but also chondrocytes and adipocytes (Friedenstein et al., 1970, 1974). However, the full extent of the potential of those cells remained largely unexplored during that era (Fig. 1.1).

The term "mesenchymal stem cells" (MSCs) and the concept that a clonogenic stromal cell could exist as a distinct class of bone marrow stem cell, independent of the hematopoietic stem cell, was initially coined by Friedenstein; however, it took nearly 20 years and significant advances in the definitions and characterization of MSCs to accurately define and name the cells themselves as "mesenchymal stem cells" and their critical biological characteristics (Caplan, 1991). Although Friedenstein laid the groundwork for the concept of MSCs, it was not until Arnold I. Caplan coined the term "mesenchymal stem cells" in 1991 that the field began to grow elsewhere (Fig. 1.1).

Caplan (1991) mentioned in the abstract of his seminal article that:

> "Bone and cartilage formation in the embryo and repair and turnover in the adult involve the progeny of a small number of cells called mesenchymal stem cells. These cells divide, and these cells' daughters become committed to a specific and characteristic phenotypic pathway, a lineage with steps that are discrete, and finally, end-stage cells integral to the manufacture of a unique tissue type—cartilage or bone, for example. The local curing (extrinsic factors) and the genomic potential (intrinsic factors) interact at each lineage step to control the rate and characteristic phenotype exhibited by the cells in the emerging tissue. The study of these mesenchymal stem cells, whether isolated from embryos or adults, provides the basis for the emergence of a new therapeutic technology of self-cell repair. The isolation, mitotic expansion, and site-directed delivery of autologous stem cells can govern the rapid and specific repair of skeletal tissues" (Caplan, 1991).

However, Caplan's terminology inadequately characterized the multipotent nature of these cells, which can differentiate into various cell types contributing to connective tissue development in multiple organs. The investigation of MSCs, derived from both embryonic and adult tissues, laid the groundwork for a novel therapeutic approach centered on self-cell repair, as proposed by Caplan in 1991 (Caplan, 1991). This spurred extensive research into MSCs applications in regenerative medicine and tissue engineering due to their ease of culture and manipulation for in vitro differentiation (Kfoury & Scadden, 2015).

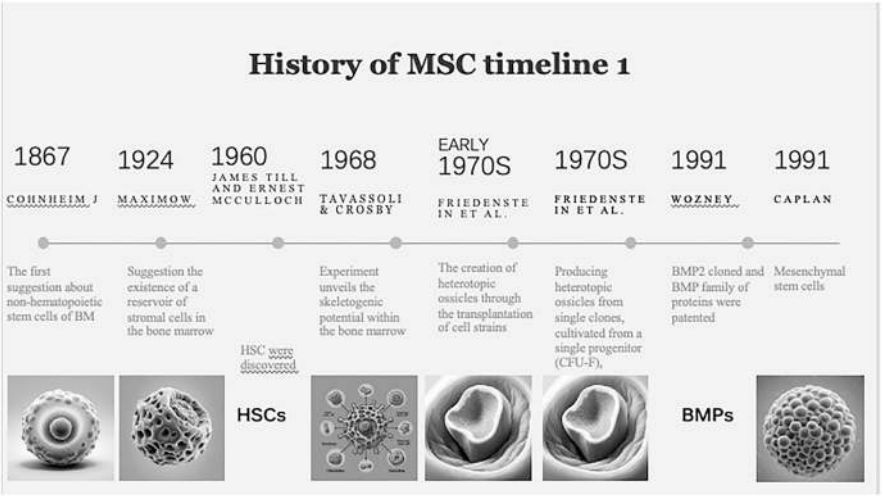

Fig. 1.1 Historical perspective of mesenchymal stem cells (1)
1867—Bone marrow origin of fibroblasts: Julius Cohnheim, in 1867, proposed that the bone marrow is the source of fibroblasts. This postulation implied that cells residing in the bone marrow produce not only blood cells but also an array of connective tissue cells (Kfoury & Scadden, 2015).
1924—A reservoir of stromal cells in the bone marrow: Alexander Maximow suggested that there is a reserve of stromal cells in the bone marrow, which is probably pluripotent and can give rise to various connective tissue cells (Kfoury & Scadden, 2015).
1968—Experiments on skeletogenic potential: Tavassoli and Crosby examined the skeletogenic potential. Their research showed that bone marrow cells form a skeletal tissue (Tavassoli & Crosby, 1968).
1970—Confirmation of stromal progenitors in the bone marrow: Friedenstein and his colleagues established the presence of stromal progenitor cells in the bone marrow, which were defined as stem cells that can differentiate into virtually all tissue types (Friedenstein et al., 1970).
1974—Single progenitor multipotent precursor of skeletal tissues: Friedenstein et al. demonstrated that clones originating in a single progenitor cell were capable of forming skeletal tissues, implying these cells are multipotent (Friedenstein et al., 1974).
1988—Osteogenic properties of marrow-derived stromal cells: Owen and Friedenstein demonstrated that the cells derived from bone marrow stroma possessed osteogenic (bone-forming) properties (Owen & Friedenstein, 1988).
1991—Mesenchymal stem cells: Arnold Caplan named these cells as “mesenchymal stem cells” (MSCs) (Caplan, 1991).

1.2 Bone Marrow-Derived MSCs Are Bona Fide Stem Cells

It was first recognized that the postnatal mammalian (human) bone marrow contains a population of non-hematopoietic stromal cells with properties of both multipotency and self-renewal (Owen & Friedenstein, 1988). Their in vivo transplantation experiments provided evidence for the serial skeletogenic potential of uncultured bone marrow tissue, non-hematopoietic (stromal) cells, CFU-Fs, a subset of stromal cells that initiate clonal growth ex vivo, and most recently perivascular cells, which were described based on phenotype. The combined use of in vivo transplantation with serial studies of in vitro adherence has allowed the definition of stem cells based on both multipotent and self-renewing properties. Friedenstein and

co-workers used in vivo transplantation of bone marrow-derived MSCs. Transplantation experiments of this kind, which are used in defining stem cell properties, almost invariably specifically refer to the transfer of cells from one organ to another for observing the behavior of these cells, their potential for differentiation, and competence for regeneration of the tissue. Hence, heterotopic transplantation represents an ideal model system by which to demonstrate that a cell strain has the intrinsic ability to underpin histologically verifiable skeletal tissues in the absence of external skeletogenic cues that are placed ex vivo or originating from cell grafting within the bone (orthotopic transplantation). The definition of multipotent, self-renewing bone marrow-derived "MSCs" has been derived from a progressive series of in vivo experiments refined over several decades (Bianco et al., 2013). Initially, Tavassoli and Crosby (Tavassoli & Crosby, 1968) investigated the transplantation of whole bone marrow fragments to extraosseous locations, in which, over time, they could detect the formation of heterotopic miniature bone structures that included the hematopoietic microenvironment (ossicles). Later, guinea pig bone marrow fibroblast colonies were cultured in monolayers later by Friedenstein et al. (Friedenstein et al., 1970). Heterotopically transplanted colony-forming units (CFUs) of adherent non-hematopoietic cells from guinea pig bone marrow into ossicles provide, therefore, the first in vivo evidence for the existence of assayable stromal progenitors within the bone marrow. This is only because the functional characterization of stem cells requires the creation of a heterotopic "ossicle." As a result, in this respect, the definition of functional properties of all types of stem cells can only be established by in vivo transplantation experiments. Transplantation in vivo of MSCs derived from the bone marrow gives rise to a miniature bone organ, displaying suitable histological and architectural characteristics that allow the engraftment and development of hematopoiesis from the host animal (Bianco et al., 2013). These cells thus represent actual stem cells for all tissues present within the skeletal segment, including the bone tissue, cartilage, adipocytes, fibroblasts, and hematopoiesis-supporting stroma. These are produced in proper transplantation assays, and they derive from a single cell if one initially isolates a single cell and uses it to make a transplantable clonal progeny. However, the self-renewing capacity of MSCs derived from the bone marrow could only be substantiated with proper in vivo evidence in the early 2000s (Méndez-Ferrer et al., 2010; Sacchetti et al., 2007).

The isolation and cultivation of human MSCs did not take off until the early 1990s, marking a pivotal moment in stem cell research (Fig. 1.2). When cultured in vitro, MSCs reveal their complex nature, presenting a mix of multipotent stromal cells. These cells include both stem and progenitor cells with varying levels of differentiation potential, alongside differentiated cells from various mesenchymal tissues. The pioneering work of Haynesworth, Lazarus, and Pittenger confirmed the presence and versatile differentiation capacity of human MSCs, sparking a significant interest in their therapeutic possibilities (Haynesworth et al., 1992; Lazarus et al., 1995; Pittenger et al., 1999).

The identification of MSCs from the bone marrow paved the way for discovering other sources of these versatile cells. Today, MSCs can be isolated from multiple tissues, each offering unique characteristics and potential uses. While the bone

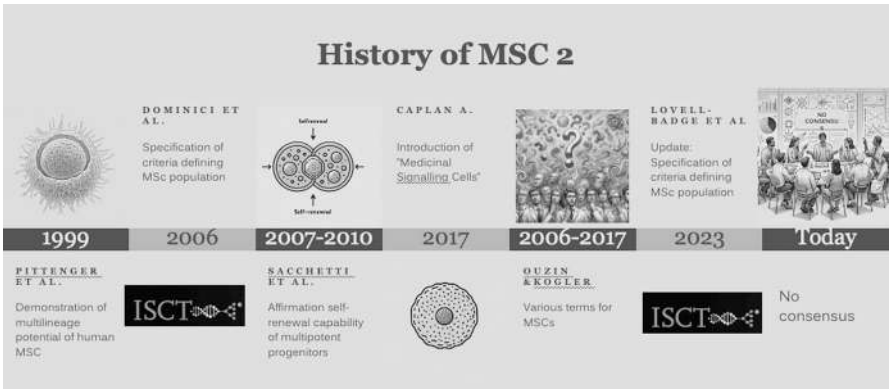

Fig. 1.2 Historical perspective of mesenchymal stem cells (2)
1999—Multilineage potential of adult human mesenchymal cells: Pittenger and team proved that the adult human mesenchymal cells are capable of differentiating into many cell types like the bone, cartilage, fat, and other connective tissues (Pittenger et al., 2019).
2006—Criteria for MSCs population: General classification of MSCs was set up according to standards in identifying and classifying the MSCs by Dominici et al. (Dominici et al., 2006).
2007–2010—Self-renewal capacity of multipotent progenitors: It has also been shown that there is a self-renewing capacity of cells in multipotent progenitors by Sacchetti et al. (Sacchetti et al., 2007). This was performed in studies with cells derived from a single parent in the bone marrow.
2017—Medicinal signaling cells: Arnold Caplan renamed MSCs "medicinal signaling cells" to emphasize their healing capabilities (Caplan, 2017).
2006–2017—Promoting awareness of different MSC terminology: Several researchers in this period were using various terms for MSCs. This was a little confusing in the literature and created a poor impression (Ouzin & Kogler, 2023).
2023—ISCT updated specification of criteria defining MSC population (Lovell-Badge et al., 2021).
Today—there is still no consensus on the terminology and definition of MSCs.

marrow and adipose tissues remain the primary sources for studying MSCs' regenerative capabilities, cells from the dental pulp, synovium, synovial fluid, Wharton's jelly, cord blood, placenta, muscle, and menstrual blood are also used (da Silva Meirelles et al., 2006).

In other organisms, the in vitro detection of stem cells, mesenchymal or not, hinges on their ability to attach to plastic surfaces. Initially, MSCs were identified in vitro by their adherence to plastic surface, expression of specific cell surface markers, and their ability to differentiate into various cell types such as chondrocytes, osteocytes, and adipocytes (Caplan, 1991). Typically, the digested or minced tissue is treated with an appropriate medium, allowing individual cells that can adhere to plastic to divide and form colonies (clonogenicity) or spread across the plastic surface as a dense group (confluency). After the first passage, further passaging increases the cell number, and characterization usually confirms that nearly all these proliferating cells are stem cells. However, to demonstrate their multidifferentiation capacity in vivo, these characterized stem cells are rarely subjected to hetero-transplantation experiments.

The journey of discovering MSCs has been marked by significant scientific milestones and conceptual breakthroughs. Recognizing the existence of stem cells for

skeletal tissues led researchers to focus on their established physiological roles. This transition involved defining genuine stem cells capable of differentiating into all bone tissue cells within the bone marrow stroma to identifying MSCs in almost all body tissues. However, an overlooked aspect was that the in vivo potential of bone marrow-derived MSCs is system-restricted and cell-autonomous. They generate skeletal tissues but not non-skeletal tissues and do not require external factors like BMPs to initiate a skeletogenic transcriptional program (Bianco et al., 2013).

The fundamental science behind the conceptual framework of MSCs portrayed them as committed multipotent progenitors for skeletal tissues and skeletal tissues only. Their residence is in the bone marrow and not elsewhere or everywhere. Most importantly, it defines them as locally transplantable, but not necessarily systemically transplantable (Bianco et al., 2013). Bianco et al. reported that this precise concept, along with its underlying scientific basis, was misinterpreted in the 1990s (Caplan, 1991) to develop a fundamentally different idea. This new concept gained widespread acceptance and became inextricably linked to the term "mesenchymal stem cells," which is now universally used. For a more comprehensive explanation, please refer to Bianco et al. (Bianco et al., 2013).

The reason stem cells have acquired so much attention in society is the belief that, due to the cell differentiation hierarchy in developmental biology, adult stem cells exist in almost all organs of the body to maintain tissue integrity and facilitate repair and healing in cases of injury or disease, even though they were previously thought not to be present in the heart and brain. This belief has evolved into the notion that stem cells can be obtained for repair or healing in almost any tissue, thanks to the isolation of these stem cells observed during the embryonic period using Friedenstein's method and their ability to be multiplied using Caplan's method.

1.3 Nomenclature

Along with the conceptual understanding of MSCs, there is also a considerable debate within the field about the appropriate terminology for plastic-adherent cells, commonly referred to as MSCs (Fig. 1.2). The International Society for Cellular Therapy (ISSCR) suggests naming these human fibroblast-like, plastic-adherent cells "multipotent mesenchymal stromal cells," no matter where they come from. They reserve the term "mesenchymal stem cell" for those that meet specific stem cell criteria (Horwitz et al., 2005). The ISSCR also set out minimal criteria for defining these cells, which include sticking to plastic and expressing certain markers (CD73, CD90, and CD105) while lacking others (CD11b or CD14, CD19 or CD79α, CD34, CD45, and HLA-DR), along with the ability to differentiate into osteoblasts, adipocytes, and chondroblasts in vitro (Dominici et al., 2006).

However, as research has progressed, especially with MSCs from tissues other than the bone marrow, these criteria have needed adjustments. The term "mesenchymal stem cells" (MSCs) has gained widespread recognition and is currently the most commonly used, even though it raises questions about how truly "stem" these

cells are (Ouzin & Kogler, 2023). Nowadays, alternative names such as multipotent stromal cells, marrow stromal cells, mesodermal stem cells, and mesenchymal stromal cells, among others, are also used in place of MSCs. In his latest work, Caplan suggests a new name for these cells: "medicinal signaling cells," highlighting their therapeutic mechanisms after transplantation (Caplan, 2017).

1.3.1 Renaming Mesenchymal Stem Cells: The Rationale Behind Medicinal Signaling Cells

For the past 30 years, since Caplan's team first introduced mesenchymal stem cells (MSCs) (Caplan, 1991), scientists have been working hard to understand these cells from various adult tissues and their potential for tissue repair and regeneration (Fig. 1.2). Initially, MSCs were thought to work mainly by turning into different types of cells like the bone, cartilage, and fat. However, extensive research has revealed that their main therapeutic effects come from the bioactive molecules they secrete, which help modulate the immune system, promote tissue repair, and reduce inflammation (Caplan, 2017). The term "stem cells" suggests that MSCs mainly function by differentiating into other cell types, leading to unrealistic expectations about their direct regenerative abilities in patients. To address this and provide a clearer understanding of their true function, Dr. Arnold Caplan, a pioneer in MSC research, has proposed renaming MSCs to "medicinal signaling cells" to better reflect their biological roles and therapeutic potential (Wakitani et al., 2023). This suggestion comes from an evolving understanding of how MSCs work. While they were once admired for their ability to turn into osteoblasts, chondrocytes, and adipocytes, it is now clear that the therapeutic benefits of MSCs are mainly due to their paracrine signaling abilities rather than direct differentiation.

Caplan's proposal highlights the crucial role MSCs play in influencing the local cellular environment by secreting bioactive molecules like cytokines, growth factors, and exosomes. These secreted factors help in immunomodulation, anti-inflammation, and tissue regeneration by affecting resident and recruited cells. By renaming MSCs to "medicinal signaling cells," Caplan aims to emphasize this paracrine mechanism, which is key to their therapeutic effectiveness in clinical applications. This change in terminology is intended to correct misconceptions and align scientific language with the current understanding of MSC biology, promoting more accurate expectations in clinical settings (Wakitani et al., 2023).

Moreover, the term "medicinal signaling cells" highlights the therapeutic potential of MSC-derived secretomes in regenerative medicine. This includes their use in treating a wide range of conditions, such as autoimmune diseases, cardiovascular disorders, and tissue injuries. Recognizing MSCs primarily as signaling entities rather than stem cells could lead to new therapeutic strategies that utilize their secretory functions, such as cell-free therapies using conditioned media or exosome-based treatments. Therefore, Caplan's proposal is not just a change in name but a

strategic shift to foster innovative therapeutic approaches and improve outcomes in MSC-based research and applications (Galipeau et al., 2019).

However, there is still no consensus about the nomenclature and the function. A comment published in Nature in September 2018 succinctly yet comprehensively addressed many issues regarding MSCs (Sipp et al., 2018). This comment examines the controversies surrounding various populations of cells termed "mesenchymal stem cells" since the early 2000s. As we all know, despite ongoing debates about their origins, developmental potential, biological functions, and therapeutic applications, the term "MSCs" persists. While tissue-specific stem cells, with limited differentiation abilities, are prevalent in most adult tissues, the classification of cells as MSCs continues, even though evidence suggests they may represent tissue-specific cell types. Despite calls for revision or abandonment of the term, thousands of research articles referencing MSCs are published annually, and several national regulatory agencies have licensed MSC-based drugs (Sipp et al., 2018).

The comment also highlights the confusion surrounding MSCs, fueled by conflicting reports and a lack of standardized terminology. Authors reported this confusion has led to the widespread perception of MSCs as a solution for various conditions, despite limited scientific evidence. Therefore, Sipp et al. (Sipp et al., 2018) suggested that efforts to address this issue should include recommending more precise labeling and standardized analyses of gene expression and cell differentiation. However, the persistence of conflicting claims and the commercialization of MSC-based treatments underscore the need for clearer scientific understanding and regulatory oversight. Moreover, the name "medicinal signaling cells" has also been criticized. Sipp et al. argued that this term was assigned based on the cells' therapeutic potential rather than their inherent stem cell characteristics (Sipp et al., 2018).

Bianco et al. (Bianco et al., 2013) also highlighted and critiqued the misconceptions and misapplications surrounding MSCs in their scholarly article, and these rational explanations deserve to be transferred here directly:

> "The intense interest in stem cells stems from the belief that they play a pivotal role in tissue maintenance and repair throughout the body. This belief, fueled by the success of isolating and culturing MSCs, has led to widespread enthusiasm for their potential use in regenerative medicine. However, the specific mechanisms underlying the therapeutic effects of MSCs, often described as trophic, anti-inflammatory, or immunomodulatory, remain poorly defined. While some of these effects might be linked to their regulatory function as stromal cells in the bone marrow, many of the observed effects, such as immune modulation, are not unique to stem cells and can be replicated by fibroblasts. The lack of clear understanding regarding the precise mechanisms of action and the absence of validated in vivo models raises concerns about the validity of the claims surrounding MSCs' therapeutic potential. Moreover, the presumed roles of MSCs in various processes, such as inflammation, wound healing, and tissue regeneration, are often conflated. The "translational" perspective further blurs these distinctions, with "paracrine" effects attributed to MSCs in diverse injury contexts, ignoring the fact that different injuries trigger distinct cellular responses and microenvironments. The indiscriminate application of MSCs to a wide array of unrelated conditions, such as heart attacks, renal failure, and Parkinson's disease, without a solid scientific rationale, raises concerns about the validity and safety of these approaches.

> The lack of defined and reproducible models makes it difficult to determine whether the purported "paracrine" effects of MSCs are truly due to cell-cell interactions or the release of specific factors. Additionally, the clinical trials conducted thus far, often lacking a thorough understanding of the pharmacokinetics of injected cells and a strong rationale, are inadequate to address this question. The intravenous injection of MSCs, which are rapidly cleared from the bloodstream after causing endothelial damage in the lungs, raises further questions about the duration and mechanism of their therapeutic effects. In conclusion, the current enthusiasm for MSC-based therapies is not adequately supported by robust scientific evidence. A more rigorous approach, focused on elucidating the precise mechanisms of action, developing reliable in vivo models, and identifying the specific factors responsible for the observed effects, is necessary to move the field forward and realize the true potential of stem cells in regenerative medicine" (Bianco et al., 2013).

However, Caplan has advocated for a scientifically accurate and ethically sound approach to MSC research and clinical applications (Caplan, 2019). Another response came from ISSCR, acknowledging that MSCs are often misappropriated to give an illusion of legitimacy, and agrees that public websites like clinicaltrials.gov can be exploited by deceitful operations. ISSCR emphasized that culture-expanded MSCs should not be conflated with endogenous MSCs and recommend using the term "multipotent mesenchymal stromal cells" to reflect this distinction. Moreover, ISSCR believes that Caplan's efforts have been instrumental in shaping current MSC research, which focuses on their paracrine therapeutic effects rather than their ability to differentiate into multiple cell types (Galipeau et al., 2019).

Today, the academic community lacks consensus on both the nomenclature and the biological significance of these cells. Not only nomenclature but also the function of these cells is under debate. Some consider MSCs to be overly praised, while others perceive them as remarkably functional. At the time of writing the book (August 2024), a search on PubMed yields 93,762 results when using the term "mesenchymal stem cells" OR "mesenchymal stromal cells." Selecting high-quality research among this abundance is becoming increasingly challenging, akin to separating stones from rice. Effectively translating accumulated knowledge from the bench to the bedside may require an unconventional approach.

1.3.1.1 Magical Cells vs. Secreted Molecules

To try to smooth over this disagreement, here is an intriguing anecdote that relates to Caplan's initial efforts to isolate MSCs. While following established protocols for bone matrix demineralization (Urist, 1965), he surprisingly obtained stem cells, unlike others who yielded bone morphogenetic proteins (BMPs) using the same protocol (Wozney, 1992) (Fig. 1.2). Initially discouraged by the seemingly failed experiment, Caplan later recognized the significance of his discovery. He reasoned that if demineralized bone matrix could induce the accumulation of multipotent progenitors in adults, similar to embryonic limb bud cells, then there must be adult counterparts of these undifferentiated cells. This realization led him to identify and ultimately name these cells as adult MSCs (Caplan, 1991).

Upon realizing that MSCs do not possess the regenerative mechanism as claimed, it may be a twist of fate that almost 35 years later, Caplan's theory—that MSCs secrete a set of mysterious molecules—aims to explain the mechanism of MSC action and move away from the stem cell terminology. The BMP (bone morphogenetic protein, used here to represent the paracrine system) vs. stem cell paradox remains valid. It seems that there is an ongoing debate between studies showing that MSCs are effective and those explaining the mechanisms of how they work. If it is the secreted molecules rather than the cells themselves that are beneficial, the issue becomes even more complicated with the research focused on synthesizing these molecules into a drug (Tang et al., 2021). However, the scientific community appears to agree on the conceptual problems regarding MSCs. Some scientific communities emphasize the need for better scientific approaches, including omics analyses and rigorous assays, to accurately identify tissue-specific stem cells. Initiatives such as the Human Cell Atlas could provide valuable insights, but resolving the MSC identity crisis will require focused efforts by stem cell biologists and regulatory bodies. Furthermore, scientific societies and clinical trial registries are urged to adopt stricter standards for MSC research and terminology while encouraging researchers to rethink their use of the term (Sipp et al., 2018).

At this juncture, the trajectory of biology and medicine has bifurcated into two distinct approaches: one focused on cells and the other on secreted molecules. Nearly a century later, we are still striving to comprehend which holds greater significance for the development of functional, viable therapies for injuries and the ever-expanding array of diseases. The paradigm shifts in their mechanism of action indicate that they function in a paracrine manner. Perhaps it is time for divergence to transition into convergence. While divergence refers to moving or spreading apart, convergence implies coming together or meeting at a common point.

In a nutshell, there is an urgent need for the scientific communities to dispel the myth of MSCs as a homogeneous cell population and to adopt more precise terminology and rigorous scientific standards in MSC research and clinical applications. Instead, ISSCR has updated its Guidelines for Stem Cell Research and Clinical Translation in order to address advances in stem cell science and other relevant fields, together with the associated ethical, social, and policy issues that have arisen since the last update in 2016. This document focuses on the basic characterization to identify cell identity, ensure culture integrity, and promote material safety; evaluation of the state of undifferentiated cells and pluripotency, assessing cells and their developmental potentials appropriately; genomic characterization to assess genetic integrity and monitor cellular changes that may hinder interpretation of results or potentially manifest as malignant features; and enhancing the relevance and utility of stem cell-based model systems (organoids, microphysiological systems, engineered cells, etc.) in basic and preclinical research (Lovell-Badge et al., 2021).

1.3.1.2 What About Perivascular Cells, Pericytes?

Research on microvascular pericytes began to intersect with efforts to identify the inherent nature of in vivo MSCs, revealing phenotypic similarities between the two. Studies have shown that pericytes, when isolated from various human organs using flow cytometry and then cultured, exhibit the same characteristics as traditional bone marrow-derived MSCs. These similarities include identical morphology, proliferation rates, surface antigen expression, and the ability to differentiate into various cell types both in laboratory settings and within living organisms (Crisan et al., 2008). Generally, these cell types display nearly all the characteristics of MSCs when observed in the lab. They adhere to plastic, have a fibroblast-like shape, contain CFU-Fs, exhibit specific phenotypic traits, and have the potential to differentiate into three different cell types under the right conditions. This similarity raises an important question: are these cells from different origins inherently the same, or do the isolation, culture, and induction methods used in the lab make them appear identical (Javazon et al., 2004)?

This model was later broadened to include the perivascular spaces around larger arteries and veins. In these areas, the outermost layer, known as the tunica adventitia, contains a population of fibroblast-like cells believed to be MSCs (Corselli et al., 2012). The discovery of perivascular cells as potential progenitors of MSCs brings up the question of whether they perform the same roles in the body as they do in culture. RNA-seq studies have shown significant differences in gene expression between cultured perivascular cells and those found in the body, indicating that the conditions in the lab may greatly alter their characteristics. However, lineage tracking studies in mice have demonstrated that pericytes and adventitial progenitor cells do contribute to various mesenchymal lineages in the body, including adipocytes, myoblasts, and myofibroblasts (Pittenger et al., 2019).

Pittenger et al. (Pittenger et al., 2019) wrap up the topic with insightful questions:

> "Despite the presence of hundreds of billions of pericytes associated with the extensive network of capillaries and other blood vessels in the human body, the overall effectiveness of these cells in repairing or regenerating tissues in vivo seems surprisingly low. Only about one in ten perivascular cells yields MSCs in culture. This discrepancy between the strong potential shown by in vitro cultured MSCs from perivascular tissue and their modest endogenous role in vivo suggests a need for further research. Specifically, investigations into the clonal selection and gene expression changes that occur during their establishment in vitro could help understand and enhance the molecular control of their reprogramming into stem-like reparative cells in situ. Additionally, it is possible that perivascular MSCs are engaged in critical tissue functions that prevent their mobilization. Once these cells are harvested and cultured in vitro, they may be free to take on different roles. In the kidney, for example, pericytes serve various functions, including as mesangial cells in glomeruli and renin-secreting cells in afferent arterioles, yet they still yield 'MSCs' when purified and cultured" (Pittenger et al., 2019).

1.4 The Developmental Origin of MSCs

Natural stem cells in the body play a vital role in growth, maintenance, and repair, enabling the body to adapt and regenerate over time. Vertebrate stem cells possess several essential traits that distinguish them from differentiated cells, which are crucial for their unique role in maintaining and repairing tissues. These key traits have been suggested thus far:

- Stem cells remain unspecialized, allowing them to develop into various cell types: undifferentiated nature.
- They can replicate themselves, ensuring a continuous supply of stem cells: self-renewal.
- Stem cells have the potential to differentiate into multiple cell types: plasticity.
- They express certain specific markers that identify them as stem cells.
- Stem cells can repair and regenerate damaged tissues.
- Their function and maintenance are influenced by the surrounding microenvironment: niche interaction.
- Stem cells are persistent and active throughout an organism's life.
- They can contribute to the regeneration of various tissues: versatility across tissue types.

These defining characteristics collectively enable vertebrates to maintain homeostasis, repair injuries, and adapt to changing physiological needs throughout their lives (Cavaleri, 2006).

The ontogeny and developmental origins of MSC remain entirely unexplored within the field of MSC biology (Javazon et al., 2004). It is unclear whether MSCs resemble the stem cells that play significant roles during development. The temporal sequence of events in early human mesenchymal tissue development is unique and distinct from the repair processes occurring in adult tissues much later in life. While insights concentrated from early development may offer potential avenues for utilizing cultured MSCs in adult tissue repair and regeneration, the specific nature of this contribution remains to be elucidated (Pittenger et al., 2019). The development of MSCs during embryonic stages remains an unresolved topic. The embryological development of mesenchymal tissues is exceedingly complex, yet cellular differentiation in the developing organism appears to be deterministic; that is, the process is predictable and traceable. It is now known that the assumption that MSCs used in repair and regeneration would follow the fate of embryonic stem cells is incorrect. The notion of whether in vitro cultured MSCs will retain their "natural" identity stems from the belief that these cells, even when cultured, are thought to be natural stem cells. Furthermore, the designation of these cells as "mesenchymal" does not align with embryology (Tam & Trainor, 1994; Tani et al., 2020).

Fetal-derived stem cells from various lineages, including neural, hematopoietic, and myogenic, have shown the remarkable ability to multiply and differentiate into different cell types. Early developmental mesenchymal cells, which circulate in the body, may contribute to the stromal compartments of hematopoietic and other tissues. However, it remains unclear whether a multipotent or tissue-resident stem cell

derived from this early population exists throughout life and actively participates in tissue repair and regeneration. There is a growing evidence for the presence of a broadly multipotent or pluripotent cell in the early gestational fetus that might persist into adulthood. Yet, the specific identity of this cell and its connection to established adult populations of cultured MSCs are still not fully understood. Additionally, the lineage relationships among various embryonic, fetal, and adult pluripotent and multipotent progenitor populations require further clarification. It is also uncertain whether this cell resides within tissues or is mobilized from the bone marrow in response to injury or perhaps both (Javazon et al., 2004). The exact regenerative processes of innate stem cells remain incompletely comprehended.

Innate stem cells can be classified by their origin, potential for differentiation, as well as by their progeny. Developmental potential classifies stem cells as totipotent, pluripotent, multipotent, and unipotent. Human development initiates with the fertilization of an egg by a sperm, resulting in the formation of a single totipotent cell known as a zygote. This totipotency represents the highest level of differentiation potential, enabling cells to contribute to the formation of both embryonic and extra-embryonic structures. In the initial hours following fertilization, this zygote undergoes division, yielding identical totipotent cells with the potential to later differentiate into any of the three germ layers (endoderm, mesoderm, or ectoderm) or cells composing the placenta (cytotrophoblast or syncytiotrophoblast). Upon reaching the 16-cell stage, the totipotent cells within the morula, blastomeres, can give rise to all embryonic and extra-embryonic tissues with the suitable maternal support. Subsequently, they begin to differentiate and form either the inner cell mass of the blastocyst or the outer trophoblasts. It takes approximately 4 days, and then inner cell mass of blastocyst becomes pluripotent. The most well-known pluripotent cells are embryonic stem cells; they form all cells of all somatic and germ cells. Following the differentiation of human embryonic stem cells into a specific germ layer, they transform into multipotent stem cells with restricted potential, specifically confined to the cells within that germ layer (Cavaleri, 2006).

The progression from embryonic development to adulthood is perceived as a continuous spectrum characterized by diminishing potencies over time. Waddington's epigenetic landscape would be a good example for illustrating hierarchical family of tree of cells having the stepwise nature of commitment (Fig. 1.3) (Yildirim & Huang, 2018).

This continuous spectrum, characterized by diminishing potencies over time, is evident within the hematopoietic system. Unlike most systems, hematopoiesis features a well-defined hierarchical tree with stepwise lineage commitment. In this system, several methods have been developed to trace the fate of progeny cells. For example, to analyze hematopoietic cell lineages, bone marrow cells are first infected in vitro with a retroviral vector and then transplanted into lethally irradiated recipients. DNA probes are subsequently used to track the progeny of individually infected cells across various hematopoietic and lymphoid tissues within the host. These experiments demonstrate that individual hematopoietic stem cells are multipotent, capable of generating the full spectrum of blood cell types, both myeloid and lymphoid, as well as self-renewing to produce new stem cells (Cheng et al., 2020) (Fig. 1.4).

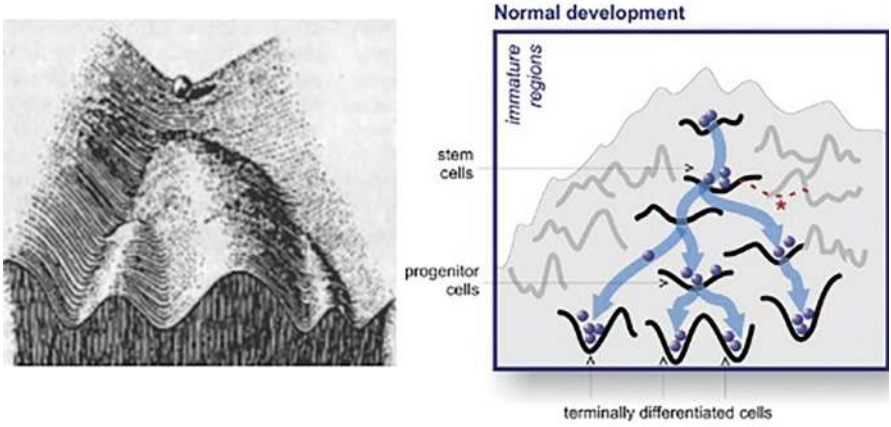

Fig. 1.3 Waddington's "epigenetic landscape". (Reproduced from Yildirim and Huang (2018) with the permission of the publisher)

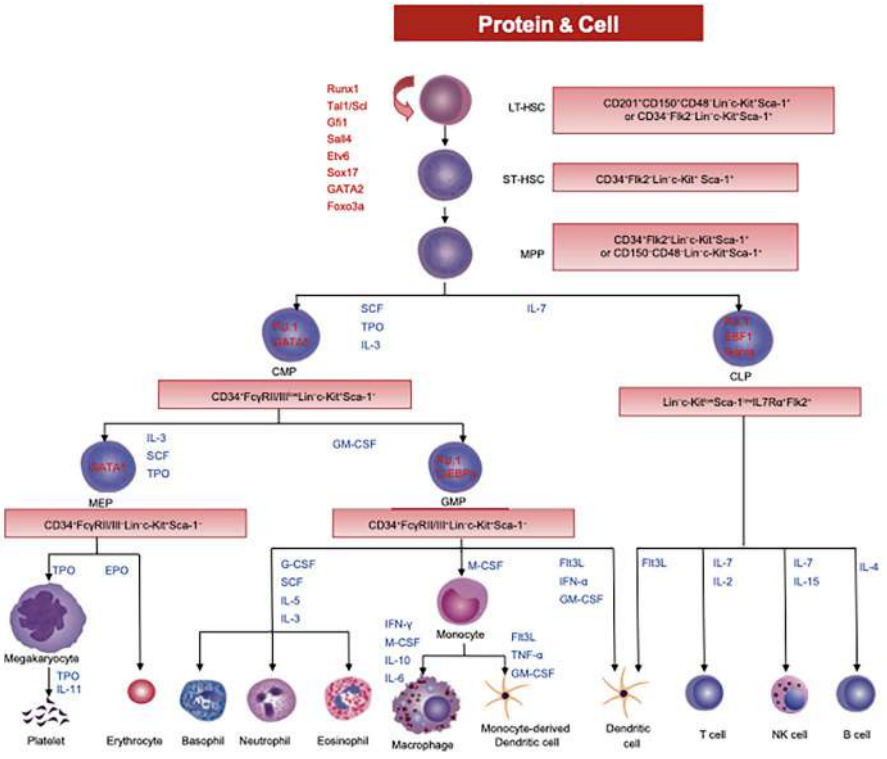

Fig. 1.4 The classical hematopoietic hierarchy. (Reproduced from Cheng et al. (2020) with the permission of the publisher)

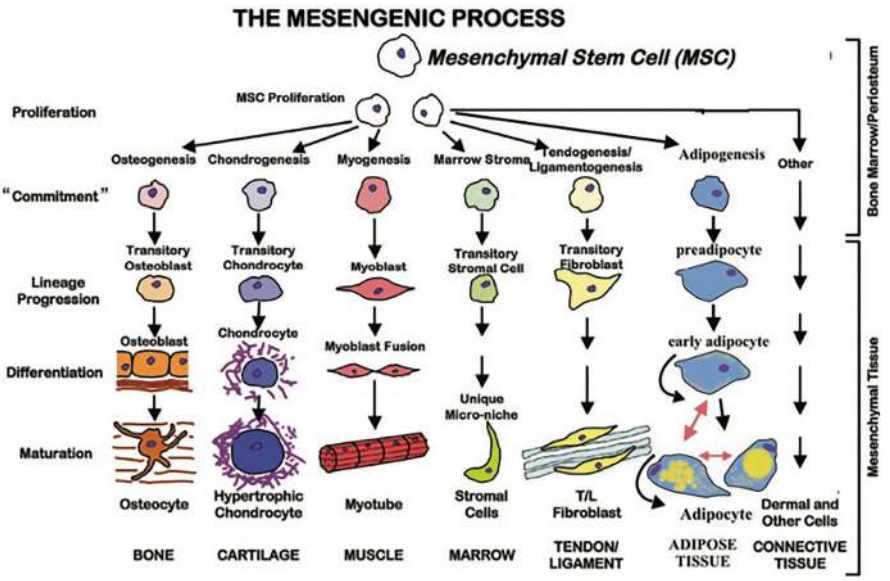

Fig. 1.5 The mesengenic process. Hypothesized scheme of a multipotent MSC self-renewing and having the capacity to be induced into several mesenchymal lineage pathways resulting in the formation of definitive tissues such as the bone, cartilage, muscle, etc. (Reproduced from Caplan (2010) with the permission of the publisher)

There has been a significant progress in understanding hematopoiesis, which has highlighted the complexity of hematopoietic differentiation. Notably, the heterogeneity within HSC and progenitor populations and the hierarchical models have been revised by novel approaches, primarily in mouse systems. While the model shown in Fig. 1.4 represents the ideal hierarchy to date, evolving perspectives have deepened our understanding of hematopoiesis and underscored the intricate nature of hematopoietic differentiation (Cheng et al., 2020).

In addition to the hematopoietic system, a multipotent stem cell that gives rise to committed progenitor cells has been primarily detected only in the bone marrow (Bianco et al., 2013). Caplan's famous figure (Fig. 1.5), which explains the mesengenic process, was so similar to the hematopoietic stem cell hierarchy (Fig. 1.4). The logic presented in Fig. 1.5 has been applied to both in vitro and in vivo assays of MSCs, and thousands of tissue engineering studies have been reported thus far. Despite the common understanding of a gradual decline in cell potency over time, the suggested pattern is not observed in MSCs. Additionally, the term "mesengenic" doesn't accurately reflect these cells' developmental origin.

1.4.1 Mechanisms Involved in the Therapeutic Properties of MSCs

The cellular and molecular basis for the therapeutic actions of MSCs has been well-reviewed by several researchers (Abbasi et al., 2023; Liu et al., 2023; da Silva Meirelles et al., 2009; Mishra et al., 2020; Mohamad Yusoff & Higashi, 2023; Naji et al., 2019; TomyTomcy & Sindhu, 2024; Yang et al., 2023; Yuan et al., 2023). We will address this subject when discussing dental pulp-derived mesenchymal stromal cells. However, here is a brief list of the proposed factors for the therapeutic actions of MSCs:

1- Homing
2- Functions exerted by soluble factors:

   a. Anti-apoptosis
   b. Immunomodulation
   c. Anti-scarring
   d. Providing assistance for the expansion and specialization of stem and progenitor cells within the local environment
   e. Angiogenesis
   f. Chemoattraction

3- As therapeutic agents for cancer (da Silva Meirelles et al., 2006, 2009)

While we have some understanding of the mechanisms behind the therapeutic effects of MSCs, more research is needed to optimize treatment strategies for graft-versus-host disease (GvHD) and other conditions. One approach is to precondition cultured MSCs with soluble factors like interferon-gamma (IFN-γ), which may boost their effectiveness in treating certain immune disorders. Additionally, exposing cultured MSCs to low-oxygen (hypoxic) conditions before administration could enhance their therapeutic potential. Research into enzymes that can efficiently harvest cultured MSCs while preserving crucial molecules for targeting injury sites could also provide therapeutic benefits. Finally, developing technologies to precisely target MSCs to injury sites for optimized cell delivery is a promising future direction in this field (Meirelles et al., 2009). As we will discuss some mechanisms in detail in the DPSC chapters, please refer to those articles (Fan et al., 2020; Li et al., 2022; Madrigal et al., 2014; Pittenger et al., 2019; Rezaie et al., 2022) for more information on the topic.

1.5 Conceptual Problems Regarding MSCs

Numerous studies have enhanced our understanding of MSCs using genetically modified mice. Nonetheless, due to technical and material constraints, there is limited information regarding the cellular biology of human MSCs. Investigating

the development of MSCs is crucial as it could provide valuable insights into their physiological functions.

1.5.1 Definition and Identity

A wealth of publications documents the in vitro properties of MSCs (see Javazon et al. (2004) as reference for details). At its most fundamental level, a stem cell is defined by its ability to self-renew and produce one or more types of differentiated offspring. Vertebrate stem cells are characterized by their ability to divide (either symmetrically or asymmetrically), their motility, their capacity to differentiate into multiple lineages, and their organization into multifunctional groupings (Pittenger et al., 2019).

MSCs are still primarily characterized by their in vitro expression of a limited set of cell surface proteins and their ability to undergo tri-lineage differentiation in response to stimuli. Phenotypically, MSCs express various nonspecific markers, such as CD44, CD49e, CD62, MHC I, and Sca-1, but lack hematopoietic and endothelial markers like CD11b, CD31, and CD45. The expression of certain markers, like CD90 and CD117, can vary depending on the species, isolation methods, and culture conditions (Javazon et al., 2004). Identifying MSCs based solely on morphological or phenotypic criteria is challenging. The most reliable method for identifying MSCs is their ability to differentiate into the bone, fat, and cartilage in vitro. However, even within clonal MSC populations, there is significant heterogeneity in differentiation potential and expansion capacity. Most MSCs tend to follow an osteogenic pathway in culture (Oktar et al., 2011; Pittenger et al., 2000).

However, in vivo identity and precise functional roles of MSCs in adult tissue homeostasis are still not fully understood, leading to ambiguity about their true nature (Barberi et al., 2005; Nombela-Arrieta et al., 2011). The primary challenge in MSC biology is the difficulty in prospectively isolating MSCs from the tissue, characterizing them, and observing their biological properties in a relatively unaltered state (Javazon et al., 2004). As we discussed above, there is an ongoing debate about the defining characteristics of MSCs, including their potency and self-renewal capabilities, causing some researchers to question the validity of the MSC concept itself (Bianco et al., 2013; Sipp et al., 2018). Different isolation and culture methods lead to heterogeneous cell populations with varying characteristics, making it difficult to compare results across studies and hindering standardization (Pittenger et al., 2019).

1.5.2 Microheterogeneity, Temporal Stochasticity, and Diversity at the Single-Cell Level

Populations of cultured stem cells are not homogenous; they exhibit microheterogeneity, temporal stochasticity, and diversity at the single-cell level (Pittenger et al., 2019). The heterogeneity of MSCs is evident in their various modes of action,

including proliferation capacity, transdifferentiation, immunophenotype, and both paracrine and microvesicle mechanisms involving secretome-derived products. Differences in these mechanisms have been observed among MSCs from different sources (comprehensively reviewed by Costa et al. (Costa et al., 2021). It is still unclear today whether there is a therapeutic mode of action.

A series of processes are conducted to determine whether the isolated single cell possesses stem cell properties. While the characteristics of natural in vivo MSCs remain largely enigmatic, the desired properties of cultured MSCs are clearly outlined in the latest ISSCR guidelines (Lovell-Badge et al., 2021). However, it is not known whether these in vitro cultured cells are indeed the same as natural in vivo MSCs. The ongoing debate about the differences or similarities between natural in vivo MSCs and isolated, replanted MSCs also affects the issues of engraftment, structural organization, and cellular differentiation (Pittenger et al., 2019).

1.5.3 Source Variability and Differentiation

MSCs can be isolated and characterized from a wide range of human tissues, even menstrual blood (da Silva Meirelles et al., 2006). Variations in differentiation frequency and extent were noted among MSC cultures sourced from different tissues, highlighting potential tissue-specific influences on differentiation capacity. Despite the ability of MSCs to differentiate into diverse cell lineages in controlled environments, achieving comparable differentiation outcomes within living systems remains a complex issue (Costa et al., 2021).

1.5.4 In Vivo Behavior

The in vivo behavior of MSCs is not well comprehended. Despite thorough in vitro studies, there is a lack of detailed knowledge about how MSCs operate and integrate within living organisms, which impacts their therapeutic use. The specific roles and functions of MSCs in their natural tissues are still ambiguous. Although they are believed to aid in tissue maintenance and repair, the precise mechanisms through which they act are not completely elucidated (Javazon et al., 2004).

Understanding the cellular biology of MSCs needs a combination of several methods like transcriptomic analysis and omics. Analyzing gene expression across the entire genome can illuminate the biological characteristics of MSCs, their anticipated function in normal physiology, their involvement in disease processes, and their potential therapeutic mechanisms. However, the clinical utility of MSCs is significantly influenced by the degree to which their gene expression is affected by, and can be altered through, manipulation of culture conditions. Another crucial issue is that a wide variety of cells (such as embryonic stem cells, induced pluripotent stem cells, and fibroblasts) exhibit extensive genetic similarity, as evidenced by

overlapping microarray profiles (Y. Wang et al., 2010). Hence, genomic studies can only be valuable if they are analyzed comparatively.

In order to remember some concepts, the transcriptome refers to the complete set of RNA transcripts produced by the genome at any given time. It includes all types of RNA molecules, such as messenger RNA (mRNA), ribosomal RNA (rRNA), transfer RNA (tRNA), and non-coding RNAs. The transcriptome reflects the genes that are actively being expressed in a cell or tissue, providing a snapshot of cellular activity. By studying the transcriptome, researchers can understand how gene expression changes in response to various conditions, such as disease, environmental changes, or developmental stages. Techniques like RNA sequencing (RNA-seq) are commonly used to analyze the transcriptome, allowing for a comprehensive view of gene expression patterns. On the other hand, omics is a broad term that encompasses various fields of biological study, all of which aim to collectively characterize and quantify large sets of biological molecules that translate into the structure, function, and dynamics of an organism or organisms. "Omics" refers to a field of research within the life sciences that involves the large-scale analysis of biological molecules and processes. It encompasses various areas of study, including proteomics (proteins), transcriptomics (RNA transcripts), genomics (genes), metabolomics (metabolites), lipidomics (lipids), and epigenomics (gene regulation). This rapidly expanding, interdisciplinary field is providing unprecedented insights into biological systems, accelerating our understanding of both normal physiology and disease mechanisms (Kabbashi et al., 2024).

The study of an organism's complete set of DNAs, including all of its genes, is called genomics. This field focuses on understanding the structure, function, evolution, and mapping of genomes. Proteomics means the large-scale study of proteomes, which are the entire set of proteins expressed by a genome, cell, tissue, or organism at a certain time. Proteomics seeks to understand protein functions, interactions, and their role in biological processes. The study of the complete set of metabolites (small molecules) within a cell, tissue, or organism is called metabolomics. It provides insights into the metabolic pathways and changes under different physiological conditions. Epigenomics instead is the study of the complete set of epigenetic modifications on the genetic material of a cell, which can influence gene expression without changing the DNA sequence. This includes DNA methylation, histone modification, and RNA-associated silencing (Melby et al., 2021; Wang & Chang, 2018).

Today, genome-wide gene expression profiles are far from fully understood, making it difficult to pinpoint the mechanisms involved. Considering the enigmatic nature of epigenetics, instead of relying solely on genome-wide gene expression, we need to focus on omics. Omics might reveal the biological activities of specific, targeted cells. By integrating data from different omics fields, researchers can gain a more comprehensive understanding of biological systems and how various molecular components interact and contribute to health and disease. Omics approaches enable the identification of biomarkers for disease, the discovery of new therapeutic targets, and a better understanding of the underlying mechanisms of complex biological processes.

As a conclusion, outline the main conceptual challenges and future directions for MSC research as follows:

- Unclear definitions and functions: Establishing standardized definitions and elucidating the precise functions of MSCs across different tissues and contexts.
- Regulation and context of MSC functions: Understanding how MSC functions are regulated by their microenvironment and how this influences their therapeutic potential.
- Translational challenges: Addressing the hurdles in translating preclinical findings into safe and effective clinical therapies, including issues of scalability, manufacturing, and regulatory approval.
- Mechanisms and efficacy: Further investigating the mechanisms underlying MSC therapeutic effects and optimizing their efficacy for various clinical applications.
- Implications for therapy: Exploring the broader implications of MSC therapy, including potential long-term effects, ethical considerations, and the development of personalized treatment approaches.

There is unquestionably a significant gap between preclinical data and clinical trials today. What explains the difference between the expectations set by preclinical data and the results observed in human MSC trials? Science needs to address again to their origins, developmental capabilities, biological roles, and potential therapeutic applications. Sipp and colleagues (Sipp et al., 2018) articulated with precision that "The wildly varying reports have helped MSCs to acquire a near-magical, all-things-to-all-people quality in the media and in the public mind—hype that has been easy to exploit. MSCs have become the go-to cell type for many unproven stem-cell interventions. The confusion must be cleared up."

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Chapter 2
Dental Evolution

After a brief overview of MSCs in the first chapter, the book will delve into dental pulp-derived mesenchymal stromal cells (DPSCs). To fully appreciate the potential of DPSCs, a solid understanding of dental development and tooth structure is essential. A deeper knowledge in this area could pave the way for innovative regenerative therapies as scientists explore methods to stimulate stem cells for tooth repair and replacement.

2.1 Historical Perspectives

Evolutionary and developmental biology are highly complementary fields of research to comparative dental embryology, which emerged in the last quarter of the nineteenth century. To establish a suitable taxonomy for dental embryology, it is necessary to compare equivalent characteristics across both extant and extinct species. Histological studies of dental organs began to emerge in the nineteenth century. According to Louise J. Baume's seminal work, *The Biology of Pulp and Dentin*, the first demonstration of tubular structures in dentin occurred between 1835 and 1851. While the recognition of the pulp-dentin relationship followed these discoveries in 1852–1863, the identification of peritubular and nerve structures was not demonstrated until later, between 1863 and 1868. The ontogeny and phylogeny of dentin were defined within this framework from 1867 to 1906 (Baume, 1980).

From agnathans (jawless) vertebrates to mammals, observed modifications in dental structure revealed the evolution of mammalian dental ontogeny. Fossil teeth provided evidence for comparing relationships between extant and extinct groups, establishing the phylogeny of mammals (Rougier & Novacek, 1998). As highly mineralized appendages found in the entrance of the alimentary canal of both invertebrates and vertebrates, teeth have many functions such as processing of food, phonetic articulation, and defense in humans (Koussoulakou et al., 2009). The fact that

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teeth originated from dermal structures in vertebrates has significant implications for dental biology. These dermal structures are believed to have migrated secondarily to the oral cavity, where they subsequently associated with the bone (Butler, 1967). Actually, the ancestors of teeth were dermal appendages: scale-like epidermal structures (Fig. 2.1a). The assumption that teeth evolved from sharks (more accurately, the extinct ancestors of sharks, the Chondrichthyes, cartilaginous fishes) appears to be correct (Fig. 2.1b).

Odontodes, which are superficial dermal structures located outside the mouth in jawless fish, were followed by buccal teeth that were localized to the jaw margins (Koussoulakou et al., 2009) (Fig. 2.2). Reif highlighted the similarity between teeth and the dermal denticles (placoid scales) of elasmobranchs (Reif, 1978). Butler expanded on this idea, suggesting that teeth may have originated as skin derivatives in jawless vertebrates and subsequently migrated to the oral cavity with the evolution of jaws in gnathostomes (vertebrate with jaws) (Butler, 1995).

The "inside-out" theory proposes that teeth originated from the endoderm. According to this theory, pharyngeal teeth first developed in jawless vertebrates and later moved forward to the oral cavity as jaws evolved (Smith, 2003). However, recent research indicates that both theories may have limitations and might not be completely accurate (Huysseune et al., 2009; Jheon et al., 2013). Very recently, Cooper et al. (Cooper et al., 2023) have mapped the development of dermal denticles, comparing conserved gene expression patterns with those of developing shark teeth. Their findings reveal shared developmental gene expression across both shark denticles and sensory receptors, suggesting a common gene regulatory network (GRN) might function during the evolution and development of all epithelial-origin skin appendages. Essentially, they discovered that shark denticle development shares a significant gene expression signature with developing teeth. However, denticles have limited a regenerative potential because they lack a Sox2+ stem cell niche, which is essential for maintaining the dental lamina required for continuous

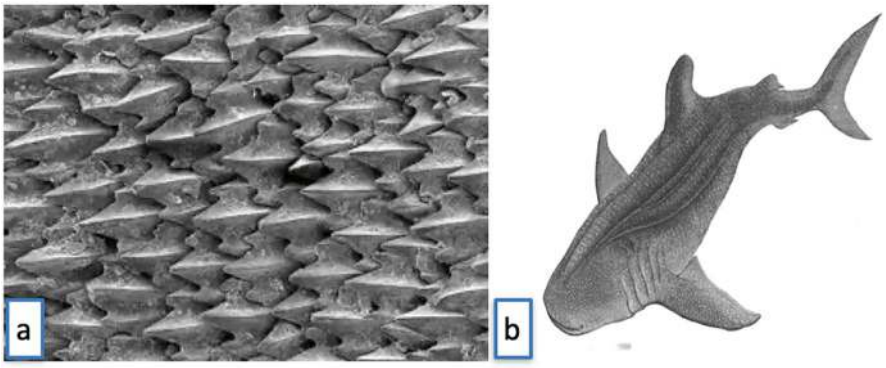

Fig 2.1 a- Representative image of dermal appendages (**a**) seen in Chondrichthyes (the extinct ancestors of sharks) (**b**)

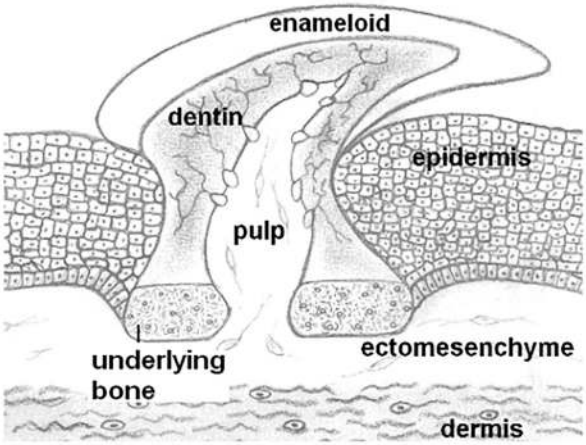

Fig. 2.2 According to Koussoulakou et al. (2009), odontodes, the ancestors of teeth, looked like placoid scales of recent sharks. Odontodes consisted of a dentine cone with a pulp cavity and covered by a hypermineralized tissue like enamel or enameloid. They were attached to the integument by a bony base. (Reproduced from Koussoulakou et al. (2009) with the permission of the publisher)

tooth replacement. The findings from Cooper et al. strongly support the "teeth outside the mouth" theory (Cooper et al., 2023).

2.1.1 Atavism

The reappearance of some teeth, which were lost over time, in birds is an interesting evolutionary concept (Chen et al., 2012). One of the central evolutionary principles known as Dollo's law indicates that an organism is unable to return, even partially, to a previous stage already realized in the ranks of its ancestors (Tomić & Meyer-Rochow, 2011). However, the reappearance of an ancestral trait in an individual organism of a species that lacks that trait, such as hind legs in whales or teeth in avian species, is considered an accident of development and is known as atavism—from the Latin word *atavus*, meaning a great-grandfather's grandfather and, thus, more generally, an ancestor. "Atavism" denotes the reappearance of an ancestral type in the anatomy of an organism. In atavism, tissues and organs appear to revert to expressing characteristics found in a distant evolutionary ancestor (Hall, 1984). The stunning, creative-constructive effect of a developmental accident, if we consider atavism one, is the production of a structure that exemplifies an organized ancestral structure (Yildirim & Huang, 2018).

Obviously, under appropriate conditions, the lost odontogenic capacity of avian ectomesenchyme can be regained (Louchart & Viriot, 2011). Odontogenesis, which we will explore further in the next chapter, requires epithelial and mesenchymal interactions. The cells are derived from the ectoderm of the first branchial arc and

the ectomesenchyme of the neural crest. Interaction allows the epithelial part to form the outer layer of the enamel, and mesenchyme is responsible for the formation of inner layers (dentin, dental pulp, attachment to the bone, bone, etc.). In 1980, Kollar and Fisher grafted 16- to 18-day-old mouse mesenchyme, which were taken from the region where the first molar teeth form, alone into the suitable space of the anterior chamber of the eyes of adult nude mice. Dentin was not developed. However, when they combined that mesenchyme with an epithelial tissue from the first and second gill arches of a 5-day-old chick embryo, they observed dentin. Thus, they proved that under appropriate conditions, the lost odontogenic capacity of avian ectomesenchyme can be regained since the oral epithelium still has an odontogenic capacity (Kollar & Fisher, 1980). This breathtaking experiment proved and convinced the evolutionary biologists of, as Gould said, "the potential structure that chick epithelium has encoded for sixty million years but has not expressed in the absence of dentin to induce it" (Gould, 1994). As we reported in our latest publication (Yildirim & Huang, 2018), regaining of the lost odontogenic potential in birds has been repeated by several experiments (Cai et al., 2009; Chen et al., 2000; Fuenzalida et al., 1990; Harris et al., 2006; Kollar & Fisher, 1980; Lemus, 1995; Mitsiadis et al., 2003; Wang et al., 1998).

Possible explanations for this rare phenomenon are the preserved odontogenic capacity in the oral epithelium or a safeguard mechanism such as gene redundancy, since knockout mutations of most teeth-related genes do not cause developmental arrest. Moreover, those mutations cannot prevent the formation of dental lamina, which can be observed in birds, although their teeth were lost over 60 million years ago (Chen et al., 2000; Koussoulakou et al., 2009). Another explanation involves the concept of latent attractors as by-products of GRN dynamics and their atavistic nature as a result of changes in the epigenetic landscape during evolution. With the concept of latent attractor as by-product of GRN dynamics and of their atavistic nature as a result of the way the epigenetic landscape changed during evolution, we can also articulate the formal and molecular principles to support these verbal assertions. Mutational rewiring of the GRN is not necessary but that environmental signals, which are complex and poorly understood and can only be replicated by transplanting entire supporting issues, can unleash the phylogenetically hidden developmental potentials. The need for a complex set of signals that come as a natural "package" in the form of the physical presence of an inducing tissue is also consistent with the need for broad combinatorial perturbations of the nodes in a network to cause an exit from an attractor state (please refer to Yildirim and Huang (2018) for more detailed explanations). Understanding the rules governing the reacquisition of the lost properties will provide a wide range of clinical and biological applications to impaired or lost teeth (Koussoulakou et al., 2009).

2.2 Early Theories of Dental Patterning

Most non-mammalian vertebrates have *homodont* dentition (all teeth of the same type). Human dentition is *heterodont* and *diphyodont*, which includes deciduous and permanent succession. While the dentition of animals with only one set of teeth throughout life is *monophyodont*, continuously replacing dentitions are *polyphyodont* (Fraser et al., 2006). The reduction in teeth number and generations (from polyphyodont to oligodont) and the increase in morphological complexity of teeth (from homodont to heterodont) represent important factors for mammalian diversification (Koussoulakou et al., 2009; Salazar & Jernvall, 2010).

2.2.1 The Field Theory

Mammalian dentition consists of the teeth that form as separate units, and the linear arrangement of the teeth at the jaw margins shows spatial repetition. Butler hypothesized that a morphogenetic field determined forms of the teeth (Butler, 1967). This concept was adapted to human dentition by Dahlberg who proposed that there was a field of influence operating on each of the tooth classes, i.e., incisors, canines, premolars, and molars (Dahlberg, 1965).

2.2.2 The Clone Theory and Reaction/Diffusion Model

Later, the clone theory of dental development was proposed by Osborn (Osborn, 1974). He postulated that an inhibitory zone exists around the developing tooth germ and that the germ can act as an attractor. This attractor acts like a pool, which attracts molecules from the surrounding tissue or tooth germ itself (Osborn, 1974). Additionally, according to the reaction/diffusion model, interactions between activator and inhibitor molecules, such as FGF8/BMP4 and ectodin/BMP4, can give repeated structures (Laurikkala et al., 2003; Neubüser et al., 1997). Moreover, the multi-attractor concept can easily be applied to the developing tooth germ (Zhou et al., 2011).

2.2.3 The Odontogenic Homeobox Code

The odontogenic homeobox code model explains how dental patterns can be generated from different domains of expression of homeobox genes in neural crest-derived ectomesenchyme (Sharpe 1995; Tucker & Sharpe, 2004). Initially developed from research on mice, which possess only incisors and molars, the genetic model

has been expanded to explain how the formation of canines and premolars might also be controlled by overlapping genetic influences (Townsend et al., 2009). Odontogenic homeobox codes are trying to explain how different tooth classes are initiated in different parts of the oral cavity in response to molecular cues and the expression of specific groups of homeobox genes. The odontogenic homeobox code aims to explain how different tooth classes are initiated in various regions of the oral cavity in response to molecular signals and the expression of specific homeobox gene groups (Suryadeva & Khan, 2015; Wang et al., 2024).

2.2.4 A Unified Model and Clinical Applications

Recently, the complementary actions of the field, clone, and homeobox code models have been proposed, and a single incorporated model for dental patterning was hypothesized (Mitsiadis & Smith, 2006). Such a unifying view can be extended into clinical settings using findings from dental patterning in individuals with missing or extra teeth (Fang & Atukorallaya, 2023; Townsend et al., 2009).

Through studies of development and mathematical models, Salazar-Ciudad and Jernvall (Salazar & Jernvall, 2010) provided new insights into the genotype-phenotype map. They developed a computational model of mammalian tooth development that integrates parameters of genetic and cellular interactions to produce a three-dimensional tooth from a simple tooth primordium. By systematically adjusting each model parameter, the researchers generated phenotypic variation and employed geometric morphometric analyses to identify and developmentally ordinate the parameters that best explain population-level variation in real teeth (Salazar & Jernvall, 2010). The paper is truly inspiring. However, it does not cover the entire process of tooth development, focusing instead on crown morphological patterns in mice and voles. If one considers that the tooth crown also includes a dental pulp chamber, the complexity exceeds what the authors have suggested. A tooth is akin to Russian Matryoshka dolls: enamel and dentin are the hard tissues covering the tooth crown externally, while the dental pulp, a soft and highly intricate connective tissue, resides inside, mirroring the shape of its host. Hence, the paper attempts to explain only the crown shape without considering the two distinct layers (enamel and dentin) and the dental pulp. Tooth development begins with crown morphogenesis, followed by root development. In the root, additional layers akin to Matryoshka dolls exist: starting from the inside out, there are the root dental pulp, root dentin, cementum (comprising several different layers), periodontal ligament, and alveolar bone attachments. Finally, the tooth erupts into the oral cavity, accompanied by the simultaneous resorption of alveolar bone and intricate morphological changes in the root. The mechanisms of eruption remain largely unknown.

Salazar and Jernvall (2010) suggested that despite the apparent complexity of gene networks governing development, the fundamental organizational principles might be fundamentally straightforward (Salazar & Jernvall, 2010). I am optimistic that these findings represent initial steps toward simulating organ development.

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Chapter 3
Tooth Development

3.1 Tooth Development

Experimental research on tooth development or odontogenesis is based very largely on the teeth of murine rodents (Butler, 1967). Pioneering work by Shirley Glasstone on rat tooth germ cultures gave detailed information about structural, histological, and self-differentiating interactions of explants (Glasstone, 1936). However, the dentition of the mouse and that of many other mammals is very different. Mice have highly specialized incisors, distinctive molar patterns, and a small number of teeth, and they do not have tooth replacement (Butler, 1967).

In vertebrate embryos, a group of cells, called neural crest (NC) cells, separate from the neural tube and migrate away from their parental epithelium to reaggregate with other cells (Fig. 3.1). In the developing embryo, almost all organs, glands, and tissues, such as the craniofacial skeleton, cornea, teeth/dentin, thyroid gland, thymus, cardiac septa, adrenal gland, melanocyte, autonomic nerve, sensory nerve, and Schwann cells, have these basic cells (Alberts, 2008; Avery & Dalton, 2016; Crane & Trainor, 2006). In addition to the unique invasiveness of NCCs, their contribution toward building the head of vertebrates has been considered to be a turning point in the evolution of the vertebrates (Gans & Northcutt, 1983).

According to Hall (Hall, 2000), the NC meets all the criteria used to define and identify a germ layer. Hall reported that the concept of three germ layers has been established for nearly 180 years, but this can be updated to include four germ layers: two primary layers (ectoderm and endoderm) and two secondary layers (mesoderm and NC) (Hall, 2008). For further and detailed information about the induction, epithelial-mesenchymal transition (EMT), delamination, migration, regions, derivatives, role in tooth development, multipotency, and stemness of NCCs, see Shyamala et al. (2015) (Fig. 3.2) (Shyamala et al., 2015).

Although NCCs are of ectodermal origin, it has been suggested to call them mesectoderm or ectomesenchyme, since they go mesenchymalization. This

S. Yildirim, *Dental Pulp Derived Mesenchymal Stromal Cells*,
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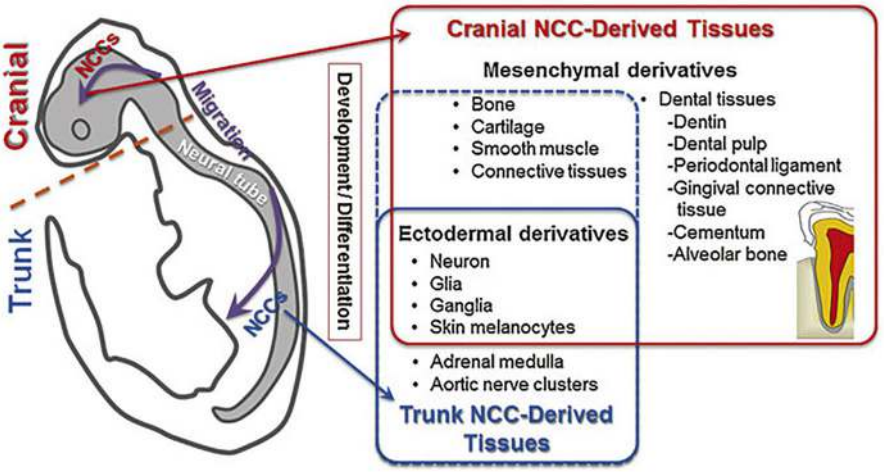

Fig. 3.1 Schematic diagram illustrating neural crest cell (NCC) location, progression, and derivatives by Niibe et al. (Reproduced from Niibe et al. (2017) with the permission of the publisher)

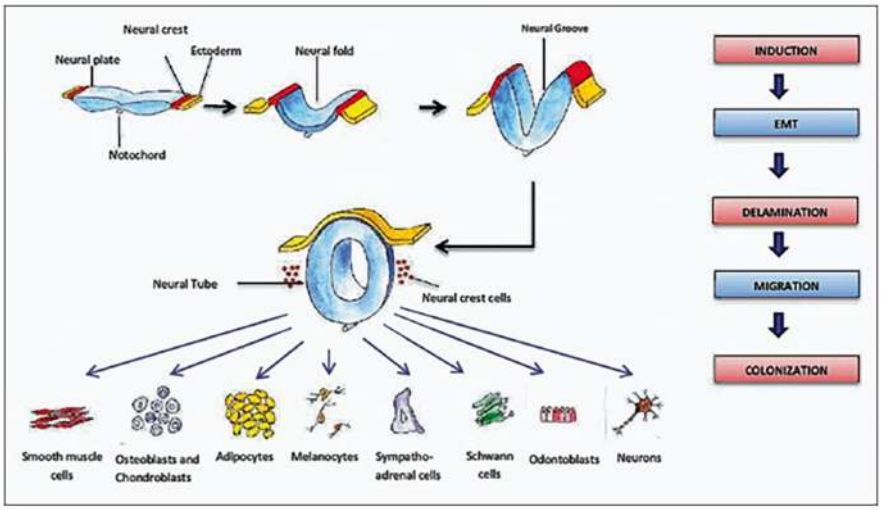

Fig. 3.2 Neural crest cells, epithelial-mesenchymal transition, delamination, and migration by Shyamala et al. (Reproduced from Shyamala et al. (2015) with the permission of the publisher)

property is important for discussions regarding MSCs, as their origin is mesenchyme, which is derived from the mesodermal germ layer. Along with the cranial skeleton and other tissues of the head and neck, odontoblasts and tooth papillae are derived from the mesectoderm or ectomesenchyme (Le Douarin et al., 2004). Oral ectomesenchymal and ectodermal inductive interactions are the earliest expressions recorded in vertebrate fossils. These epithelial (oral ectoderm) and mesenchymal

(ectomesenchyme/mesectoderm) interactions phylogenetically precede the origin of odontogenesis (Moss, 1969).

Teeth share similarities with the other ectodermal organs, such as hair, feathers, scales, beaks, and many exocrine organs, in the placode and bud stage. These similarities disappear by morphogenesis. Initiation of tooth development includes a series of sequential reciprocal inductive molecular interactions between the dental epithelium and the underlying ectomesenchymal cells (Jernvall et al., 2000). While the first signal inducing differentiation comes from the mesenchyme in all ectodermal organs, in tooth development, morphogenetic events are started by the signals from the epithelium (Jussila & Thesleff, 2012; Pispa & Thesleff, 2003). The blueprint for tooth formation is already encoded within the cells prior to the initiation of epithelial budding. In the early stages of tooth development, this prebud epithelium is responsible for initiating the process. However, this ability transfers to the underlying mesenchyme, now called postbud mesenchyme, once the bud stage is reached (Thesleff et al., 1995). According to Thesleff (Thesleff, 2018), experimental evidence from the 1960s supports this: when early tooth germs were extracted from mouse embryos and transplanted into adult mice, either in the anterior chamber of the eye, under the skin, or beneath the kidney capsule, they successfully developed into complete teeth. Several recombination studies revealed that only the very early first arch epithelium cells (occurring during the 8–11.5th embryonic days (ED) of mouse development) and ectomesenchyme (ED 12) have odontogenic potential (Lemus, 1995; Mina & Kollar, 1987).

The molecular aspects of tooth development are similar to those in the development of other organs and include epithelial-mesenchymal interactions. A plethora of molecules (approximately 300) are involved in tooth development such as fibroblast growth factors (FGFs), bone morphogenetic proteins (BMPs), sonic hedgehog (SHH), and wingless integrated (WNTs). BMP4 acts antagonistically with FGF8, and this interaction plays a role in the periodic patterning (Neubüser et al., 1997). Irma Thesleff's group from the University of Helsinki has been working on the key features of dental development. Their website, http://bite-,t.helsinki.fi/, offers a comprehensive compilation of data. Much of this information originates from previously published research, showcasing the work of researchers in this field. The website provides a source for the expressions of growth factors, receptors, signaling molecules, transcription factors, intracellular molecules, extracellular molecules, and plasma membrane molecules during the different stages of tooth development in mice, rats, humans, and other species. Those molecular signaling studies have led to the understanding that the key signaling molecules involved in organ development belong to four primary families: WNT, BMP, hedgehog (HH), and FGF. Remarkably, these signals have been conserved throughout evolution and play a critical role in regulating organ development across all animals (Thesleff, 2018). WNT signaling is widely recognized as a crucial pathway in the initiation of tooth development, playing a fundamental role in the formation of the teeth and other organs, and it has been linked with hypodontia (Yin & Bian, 2015).

Additionally, it has been found that ectodysplasin (EDA) is specifically required for the development of the teeth and other surface-derived organs such as the hair

and several glands (Mikkola & Thesleff, 2003). Obviously, the development of the teeth depends on the proper functioning of EDA and the aforementioned signaling families. Interruption of these signals can lead to halted or abnormal tooth development. Numerous mutations in the genes associated with these signaling pathways have been linked to congenital tooth absence or structural changes in dental hard tissues in both mice and humans (Balic & Thesleff, 2015). Together, most of the developmental defects in teeth usually occur as a result of mutations in genes encoding signaling molecules and transcription factors (Koussoulakou et al., 2009), such as mutations in the *PAX9* gene resulting in partial or total anodontia and mutations in *RUNX2* causing supernumerary teeth (Intarak et al., 2023; Jung et al., 2018; Peters et al., 1998; Ryoo et al., 2010).

The latest study by Thesleff's group brought attention to the glial cells as they might be the source for a developing tooth (Kaukua et al., 2014). Glial cells, which are not neurons but are indispensable for neuronal function, represent one of the most enigmatic topics in neurobiology. This study not only highlighted glial cells as a source of MSCs in the cranial region but also provided further evidence of the Karolinska Institute's groundbreaking research on teeth. One of the well-established facts about tooth development is that odontoblasts, which produce dentin, the hard tissue of teeth, and the pulp, a loose connective tissue rich in blood vessels and nerves found within the hard outer shell of the tooth, originate from NC cells that differentiate into ectomesenchyme. The researchers have shown, using clonal color-coding techniques, that peripheral glial cells, tracked through these techniques, serve as a source of multipotent mesenchymal cells that differentiate into pulp cells and odontoblasts. The teeth, which develop from the dental placode induced to form within the dental arches of the embryonic oral region, soon interact with nerves. The study demonstrated that glial-derived cells contribute to dental MSCs during tooth organogenesis. Additionally, the study suggested that multipotent Schwann cells and their precursors, genetically labeled in mice, are inactive NC-like cells. The detailed and convincing techniques used in the study revealed that the transformation of glial cells into MSCs plays a role in both the continuously growing mouse incisors and the repair responses following pulp damage. These findings suggest that a significant population of MSCs, essential for both development and repair, originates from glial cells associated with peripheral nerves. Furthermore, the results indicate that, at the earliest stages of embryogenesis, multipotent stem cells, derived from NC cells—often termed the "fourth germ layer" by Dr. B. Hall (Hall, 2000)—also originate from peripheral nerve-associated glial cells. This discovery offers critical insights into the sources of stem cells involved in organogenesis. The possibility that Schwann cells and their precursors serve as an in vivo source of NC-derived multipotent stem cells during regeneration could be a key marker in defining the limits of stem cell therapies. The implications of this study suggest that the teeth, one of the most complex organs in the cranial region, may have functions beyond merely grinding food. Furthermore, considering that not just the teeth but all organs equipped with nerves, such as blood vessels, are significant members of our nervous system could revolutionize medical thinking. An important point to note is that this research was conducted on

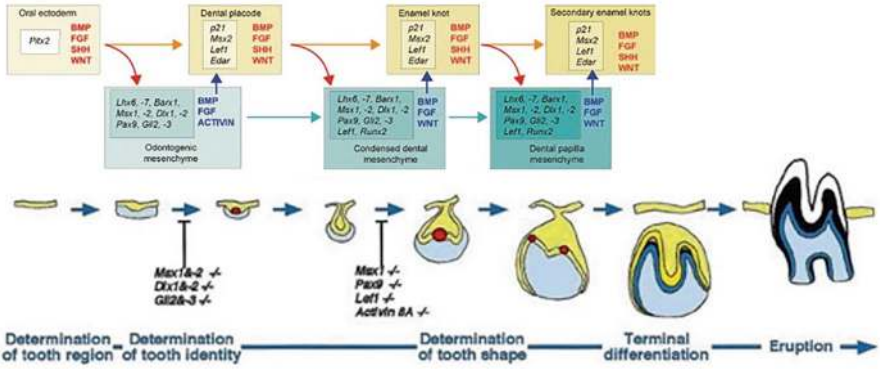

Fig. 3.3 Schematic representation of the signal and transcription factors mediating the reciprocal signaling between the epithelium and mesenchyme during advancing tooth development. The molecular cascades are shown *above* and the corresponding morphological stages *below*. The transcription factors and signals considered to be important for particular developmental stages are indicated in the squares and above the arrows, respectively. Note how the same signaling pathways are used reiteratively during advancing tooth development and how tooth development arrests in the knockout mouse experiments to the early signaling center or the enamel knot stage. Key: *yellow*, tooth epithelium; *red*, enamel knots; *blue*, tooth mesenchyme. (Modified and reproduced from (Jernvall et al. (2000) and Thesleff and Tummers (2008) with the permission of the publisher)

continuously growing mouse teeth. Whether similar findings apply to humans will be the subject of future research (Kaukua et al., 2014).

The molecular dialogue between the oral ectoderm and odontogenic mesenchyme during tooth development is very complicated (Fig. 3.3) (Jernvall et al., 2000; Thesleff & Tummers, 2008). There is a vast knowledge about the signal interchanges that are crucial to control differentiation and morphological changes and spatiotemporal expression of specific genes during teeth formation, yet little is known about the regulation of the signals (i.e., down or up) (Tucker & Sharpe, 2004). All of the data show that there is no single gene that is directly connected with ontogenesis or the lack of any specific tooth. Instead, tooth initiation and morphogenesis occur by an orchestration of numerous genetic and epigenetic factors (Jernvall et al., 2000; Koussoulakou et al., 2009).

To discover the cellular landscape of human embryonic dental development, Shi et al. (Shi et al., 2024) performed single-cell RNA sequencing and spatial transcriptome analysis on tooth germ tissues collected from aborted fetuses (17–24 weeks). Cell analysis revealed seven distinct epithelial subclusters, seven mesenchymal clusters, and additional cell types like Schwann cell precursors and pericytes. While the stratum intermedium and ameloblast lineages originated from a common outer enamel-inner enamel-ALCAM+ epithelial cell lineage, their spatial distribution was not clearly demarcated. Notably, both lineages received regulatory signals, including JAG1 and APP, from neighboring mesenchymal cells and pericytes. Moreover, pulp cell and preodontoblast differentiation exhibited four temporally distinct gene expression waves, regulated by networks involving LHX9, DLX5, and SP7, which

were in turn influenced by upstream ligands such as the BMP family. This comprehensive landscape of early human tooth development encompasses diverse spatial structures and developmental stages, offering a valuable reference for further research (Shi et al., 2024).

3.2 Stages of a Tooth Development

Each tooth passes through four morphological stages: initiation, bud, cap, and bell stages (Fig. 3.4) (Jernvall et al., 2000; Thesleff & Tummers, 2008).

3.2.1 Initiation

The initiation of the tooth begins at the end of the fifth week of human gestation and ED 10 of mouse development. A localized thickening or placodes within the primary epithelial bands, formed after about 37 days of development, initiate tooth development. In a subdivision of the primary epithelial band, the dental lamina, localized proliferative activity leads epithelial outgrowths into the ectomesenchyme.

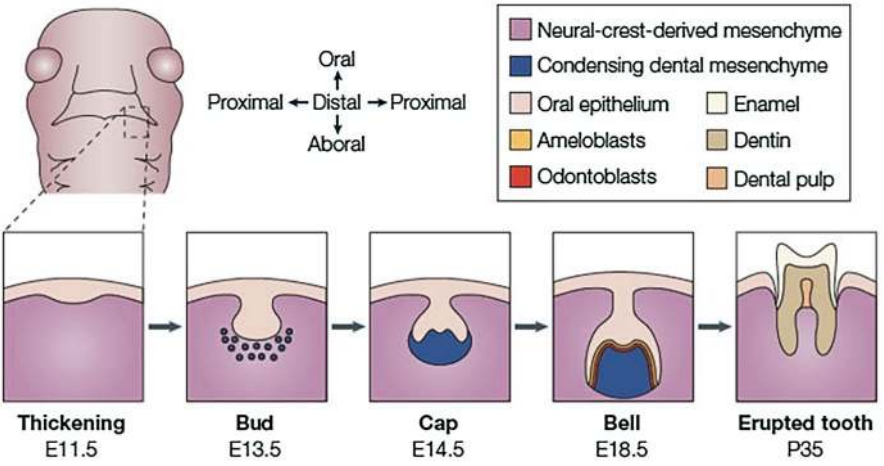

Fig. 3.4 Stages of tooth development**.** A schematic frontal view of an embryo head at ED 11.5 is shown with a *dashed box* to indicate the site where the lower (mandibular) molars will form. Below, the stages of tooth development are laid out from the first signs of thickening at ED 11.5 to eruption of the tooth at around 5 weeks after birth. The tooth germ is formed from the oral epithelium and NC-derived mesenchyme. At the bell stage of development, the ameloblasts and odontoblasts form in adjacent layers at the site of interaction between the epithelium and mesenchyme. These layers produce the enamel and dentin of the fully formed tooth. (Reproduced from Tucker and Sharpe (2004) with the permission of the publisher)

Since the underlying ectomesenchyme is more active than the epithelial cells, these ectomesenchymal cells accumulate the epithelial outgrowths soon afterward. As those cells fold, the forming structure proceeds as per the following descriptive morphological stages of tooth development: bud, cap, and bell. Folding and growth of the epithelium give the final shape of the tooth crown (Nanci, 2008; Tucker & Sharpe, 2004).

3.2.2 Bud Stage

This stage occurs between the seventh and ninth weeks of human gestation and ED 11–13.5 in mice embryos. It is represented by the first epithelial invagination into the oral ectomesenchyme. The internal part of the tooth bud contains star-like shaped, glycosaminoglycan-synthesizing stellate reticulum cells. Some cells within the stellate reticulum in mice have been identified as putative stem cells (Harada et al., 1999). Odontogenic potential is switched from the epithelium to the ectomesenchyme during the bud stage. While many ectodermal organs such as exocrine glands, hair follicles, beaks, and teeth share morphological similarity in the bud stage, different ectodermal organs become specific from the beginning of bud-to-cap transition (Jernvall et al., 2000).

3.2.3 Cap Stage

The tooth bud transforms into a cap by differential proliferation and in-folding of the epithelium (Koussoulakou et al., 2009). As the epithelial bud cells proliferate, ectomesenchymal cells condense, and morphological differences between tooth germs begin during the cap stage. In the mammalian molar tooth, the histologically distinct epithelial mass, called the enamel knot, acts as a signaling center that reiteratively influences tooth patterning. This signaling is mediated by WNT and BMP4 induction. Enamel knot cells do not show cell division, and after their transient organizing role is complete, they go apoptosis at the end of the bell stage (ED 16) (Jernvall & Thesleff, 2000; Mogollón et al., 2021). Histo-differentiation begins late in the cap stage, and in the next bell stage, the cells of the crown ameloblasts and odontoblasts are differentiated. A single layer of columnar cells, which border the dental papilla and reside inside the cap, is called the inner dental epithelium (IDE). The outer part of the cap is covered by the outer dental epithelium (ODE) (Shi et al., 2024). While the cap-shaped epithelial growth is widely referred to as the enamel organ, the condensed ectomesenchymal cells are referred to as dental papilla. The dental follicle (DF) covers the outside of these two substances. The enamel organ, dental papilla, and dental follicle constitute the tooth germ. The dental papilla is separated from the enamel organ by a basal lamina and is located between IDE and undifferentiated mesenchymal cells of the papilla. It has been shown the

expressions of Oct4, SOX-2, and MYC in dental papilla cells and dental follicle cells during in vivo tooth development and in vitro co-culture (Peng et al., 2014).

3.2.4 Bell Stage

Terminal differentiation of ameloblasts from IDE and odontoblasts from mesenchymal cells of dental papilla and the formation of two principal hard tissues of the tooth, enamel, and dentin are initiated during the bell stage. Ameloblast and odontoblast differentiation is regulated by interactions between the epithelium and mesenchyme (Hargreaves, 2002; Nanci, 2008). While dental papilla is the origin of the future dental pulp, dental follicles give rise to cementoblasts, osteoblasts, and fibroblasts. In conclusion, NC cells give rise to dentin-producing cells, odontoblasts; cementoblasts, which produce root dentin covering; osteoblasts, which participate in the formation of dental alveoli; and fibroblasts, which synthesize collagen for periodontal ligaments.

3.3 Odontoblast Differentiation

Research on amphibians has consistently shown that the cells that develop into tooth-forming cells (preodontoblasts) within the dental papilla originate from cranial NC cells. It had not been established in mammals until Ruch et al.'s seminal papers (Ruch et al., 1976, 1982). According to their researches, odontoblast's terminal differentiation occurs according to tooth-specific patterns. In the Swiss mouse molar, the terminal differentiation of odontoblasts starts at the tip of the principal cusps and progressively continues through the apical parts. The specific temporospatial pattern of odontoblast terminal differentiation has been characterized in the following steps: Preodontoblasts withdraw from the cell cycle. After the last cell division, the daughter cell that is in contact with the basement membrane elongates and polarizes. These cells start to synthesize first predentin and then dentin components (Lesot et al., 2001; Ruch, 1985; Ruch et al., 1976, 1982).

Accordingly, the steps of mature odontoblast differentiation are illustrated in Fig. 3.5:

1. Shortly after the cells of the IDE (pre-ameloblasts) at the sites of the future cuspal tips stop dividing and assume a columnar shape (pre-ameloblasts), the most peripheral cells of the dental papilla enlarge and become organized along the basement membrane (BM). Those cells now become preodontoblasts that align as a tall single layer at the periphery of the dental papilla adjacent to IDE. Between preodontoblasts and pre-ameloblasts, BM exists as the tooth's epithelial-mesenchymal interface. IDE controls those stages, and BM plays a major role as a reservoir of paracrine molecules for the continuous reciprocal epithelial-

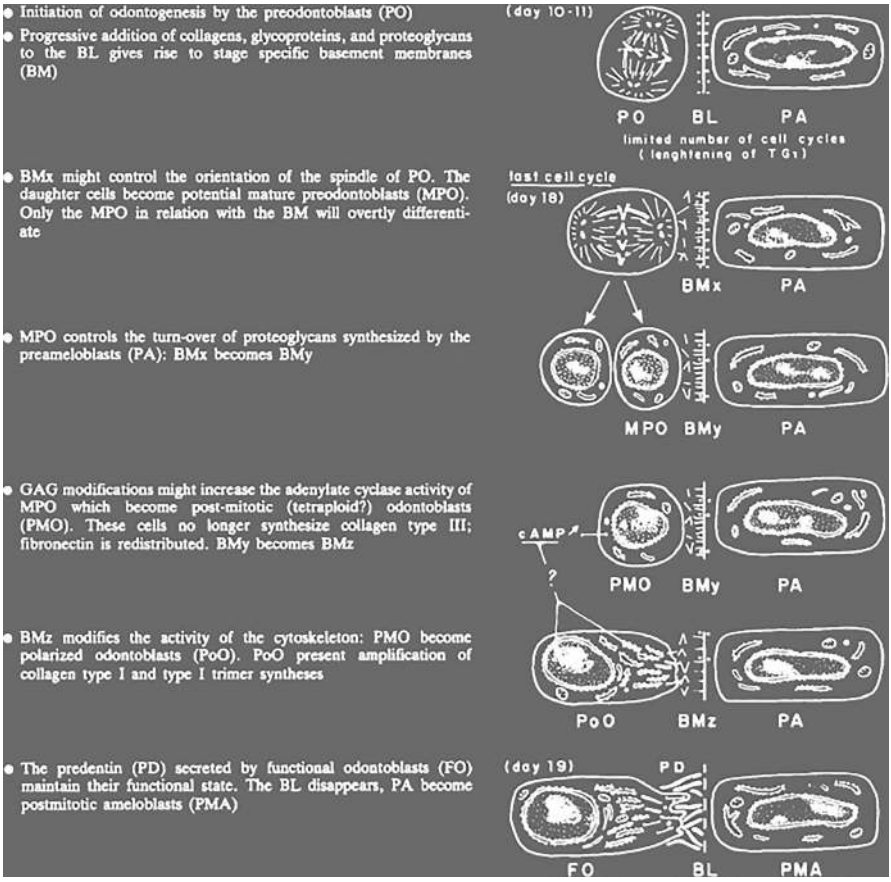

Fig. 3.5 Hypothesis concerning odontoblast differentiation. (Reproduced from Ruch et al. (1982) with the permission of the publisher)

mesenchymal interactions (Ruch et al., 1982; Timpl & Brown, 1996). Since isolated dental papillae never give rise to differentiated odontoblasts alone, the specific signals from BM are necessary to initiate odontoblast differentiation. Type IV collagen, fibronectin, tenascin, laminin, nidogen, hyaluronic acid, and heparan sulfate are the main components of BM (Lesot et al., 1981). Of note, in a mature pulp, BMs are located at the cell-connective tissue interfaces of endothelial cells and Schwann cells (Okiji, 2002).

2. From dental lamina formation to the appearance of the first post-mitotic odontoblasts, 14 or 15 cell cycles may occur. After a significant lengthening of the duration of the preodontoblast cell cycle from approximately 10–14 h, the last cell division occurs, and it is asymmetric. The mitotic spindle is oriented perpendicular to the BM during the last cell division, and at the end of the division, only

the daughter cell that is in contact with the BM will give a terminally differentiated odontoblast.
3. This cell rapidly polarizes as the nucleus takes up an eccentric basal position and shows active protein-synthesizing properties (Ruch et al., 1982).

The final stage of ameloblast development is initiated by signals from nearby odontoblasts that are actively secreting predentin matrix. Specific signaling molecules called BMPs, released from odontoblasts, are necessary for this ameloblast differentiation process (Balic & Thesleff, 2015).

3.3.1 Asymmetric Division of Preodontoblasts

Odontoblasts are post-mitotic cells, and their differentiation has many properties in common with the events seen in asymmetric cell division of stem cells (Horvitz & Herskowitz, 1992). Stem cells possess the unique ability to both replicate themselves and produce specialized daughter cells. This is achieved either by an asymmetric division, where specific factors that determine cell fate are distributed to only one daughter cell, or by positioning themselves during division so that only one daughter cell remains in contact with the niche, thus preserving stem cell characteristics (Knoblich, 2008).

For stem cells to self-renew, it has been suggested that an intrinsically asymmetric cell division whereby stem cells segregate cell fate determinants into only one of the two daughter cells by two different mechanisms occurs (Fig. 3.6) (Knoblich, 2008).

In the niche-dependent type of asymmetric cell division, the mitotic spindle of dividing stem cells orients perpendicularly to the niche surface; hence it ensures that only one daughter cell can maintain contact with the stem cell niche and retain the

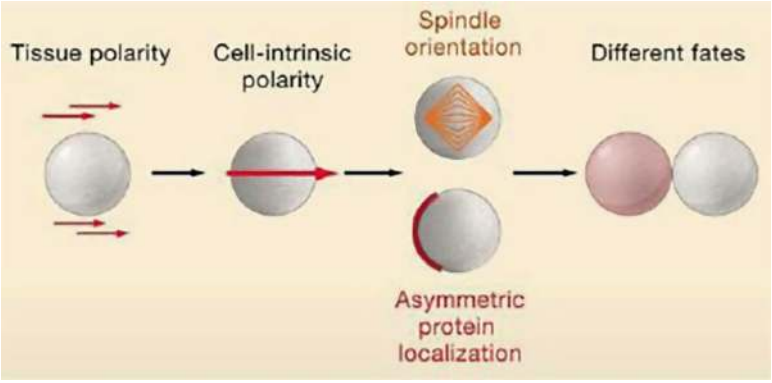

Fig. 3.6 An asymmetric cell division is defined as any division that gives rise to two sister cells that have different fates—a feature that can be recognized by differences in size, morphology, gene expression pattern, or the number of subsequent cell divisions undergone by the two daughter cells. (Reproduced from Knoblich (2008) with the permission of publisher)

ability to self-renew. Alternatively, regulators of self-renewal are localized asymmetrically during mitosis by an intrinsic mechanism, so that those regulators are inherited by only one of the two daughter cells (Knoblich, 2008; Yu et al., 2006). In this context, the orientation of the mitotic spindle during primary odontoblast differentiation could serve as a starting point for identifying stem cells within the dental pulp. Applying this information to the aforementioned scenario of odontoblast terminal differentiation, it becomes clear that preodontoblasts undergo asymmetrical division, with mitotic spindle orientation occurring after cell cycle exit. During the last division, the mitotic spindle, originally parallel to the BM, realigns to be perpendicular to BM, and only the cell in contact with the BM is able to enter into the terminal differentiation process and become a fully differentiated odontoblast (Lesot et al., 1981). Instead, the other daughter cell, which is not in contact with the BM, becomes a part of Höhl's cell layer. According to Baume (Baume, 1980) in 1896, German scientist Höhl named these cells. Subsequently, Höhl cells were believed to differentiate into odontoblastoid cells upon the death of original odontoblasts. In odontoblast terminal differentiation, the BM may function as a niche. Following asymmetrical division of the preodontoblast, the Höhl cell could potentially persist as a "stem cell" within the subodontoblastic layer or migrate into the deeper pulp, while the other cell differentiates into an odontoblast. We will elaborate on this often overlooked or neglected aspect in the fifth chapter.

3.4 Dental Papilla and Follicle

Simultaneous to odontoblast differentiation, IDE differentiates to ameloblasts that secrete enamel just before the first mantle layer of dentin is formed by odontoblasts. It has been thought that the proteins or growth factors secreted by ameloblasts have some effects on the terminal differentiation of odontoblasts, possibly by interacting with components of BM (Nanci, 2008). When dentin has formed, the enamel-producing cells assemble as a layer. Then, ameloblasts move away from the dentin leaving secreted enamel behind. Differentiating odontoblasts need signals from differentiating ameloblasts and vice versa, meaning that tooth development needs reciprocal, complex epithelial-mesenchymal interactions (Ruch et al., 1976).

The dental follicle (DF) is a dense layer of a specialized tissue derived from an embryonic ectomesenchyme. Enveloping both the enamel organ and dental papilla, it plays a vital role in tooth formation and eruption. Within the DF are cells that differentiate into a variety of cell types essential for tooth structure and function, such as fibroblasts, osteoblasts, cementoblasts, and mesenchymal progenitors (Eldeeb et al., 2024).

Single-cell RNA sequencing (scRNA-seq), a method that profiles gene expression at the level of individual cells, has significantly advanced our understanding of the diversity and behavior of cells in both healthy development and disease. Unlike conventional methods, scRNA-seq provides a detailed look at gene activity within single cells, revealing rare cell types and predicting how cells change and interact

with each other. This powerful technique has impacted various fields, including developmental biology, where it has shed light on gene expression patterns during embryo formation, leading to insights into cell fate determination and tissue development (Eldeeb et al., 2024).

A scRNA-seq study characterized the DF cells (Alghadeer et al., 2023): Cells originating from the dental mesenchyme were categorized into six distinct types: dental ectomesenchyme, dental follicle (DF), dental papilla (DP), subodontoblasts, preodontoblasts, and odontoblasts, each characterized by unique gene expression patterns. The analysis of the developmental trajectory of these cells suggested that the DP and DF, both derived from the dental ectomesenchyme, serve as potential progenitor sources. Specifically, the DP gives rise to preodontoblasts, which then mature into odontoblasts, while the DF produces subodontoblasts that have the capacity to transition into preodontoblasts (Fig. 3.7) (Alghadeer et al., 2023; Eldeeb et al., 2024).

When the first calcified matrix appears at the tip of the principal cusp, the DF is referred to as the tooth pulp. The cells of the pulp in this stage are undifferentiated mesenchymal cells, and a few collagen fibrils are seen in the extracellular matrix. The blood vessels in the dental papilla form clusters, whose position coincides with the root formation positioning. Unfortunately, there is no detailed information about angiogenesis during tooth development. On the other hand, the first nerve fibrils, as well as the vessels, approach the developing tooth during the bud-to-cap transition stage. Nerve fibrils penetrate the papilla when dentinogenesis begins. It has been assumed that the initial innervations are involved in the sensory innervation of future periodontal ligament and pulp.

The root is formed via Hertwig's epithelial root sheath, which consists of epithelial cells of the IDE and ODE. This sheath extends around the dental pulp and is almost closed except for the little opening, *apical foramen*, in the apical portion of the root. As root formation proceeds, epithelial cells influence the differentiation of odontoblasts from the ectomesenchymal cells at the periphery of the dental papilla as well as cementoblasts from the follicle mesenchyme. This leads to the deposition of root dentin and cementum, respectively. Although this describes the formation of a single root, multi-rooted teeth are formed in the same manner (Hargreaves, 2002; Nanci, 2008). While roots are forming, the supporting tissues of the tooth from the dental follicle also develop. The dental follicle gives rise to various components of the periodontium, namely, the periodontal ligament fibroblasts, the alveolar bone of the tooth socket, and the cementum. These structures also play a role during tooth eruption, which marks the end phase of odontogenesis (Hargreaves, 2002).

3.5 Formation of Permanent Dentition

Permanent dentition has the same pattern as primary dentition and the tooth germs of permanent incisors, canines, and premolars also arise from the dental lamina. However, permanent molars have no deciduous predecessors. Their dental lamina

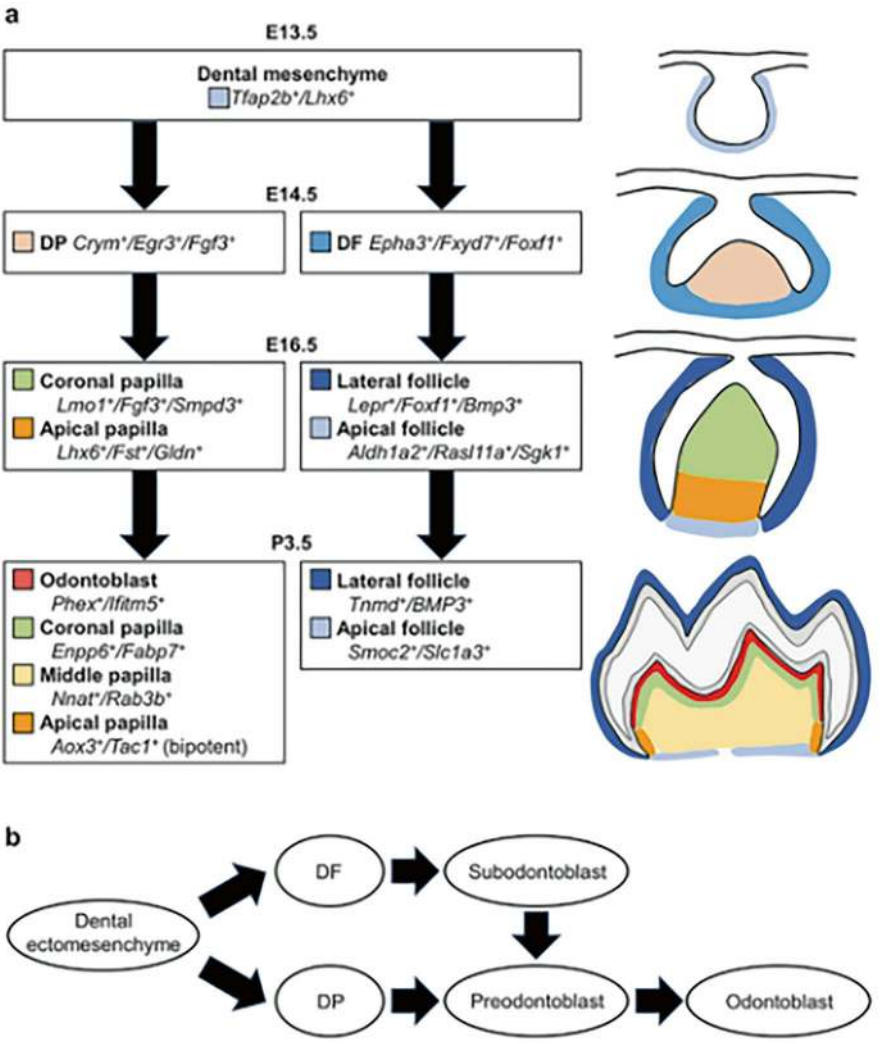

Fig. 3.7 Cellular dynamics and trajectory of the dental mesenchyme. (**a**) Cellular dynamics during mouse molar development, as presented by Jing et al. (Jing et al., 2022). At the bud stage (E13.5), the dental mesenchyme is marked by Tfap2bþ and Lhx6þ. Multiple lineage specification processes eventually generate four DP domains and two DF domains until the crown-root transition stage (P3.5). Among them, the Aox3þ/Tac1þ apical DP contains bipotent progenitor populations that give rise to odontoblasts and dental pulp cells. (**b**) Cellular trajectory proposed by Alghadeer et al. (Alghadeer et al., 2023). The dental ectomesenchyme generates the DP and DF. The DP is a major source of the odontoblast lineage. Interestingly, the DF is also predicted to be a source of subodontoblasts, which potentially differentiate into preodontoblasts. (Reprinted from Eldeeb et al. (2024) with the permission of publisher)

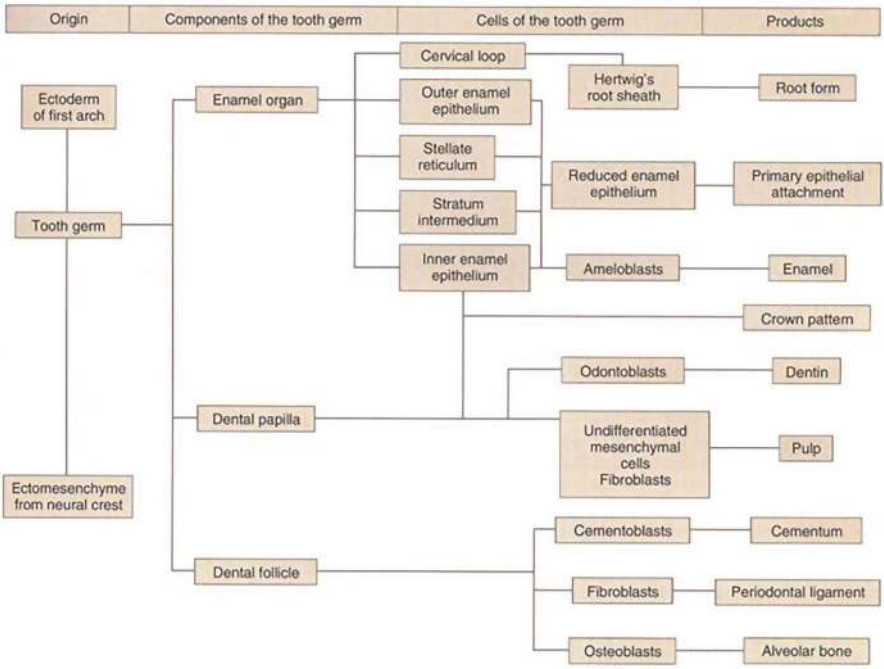

Fig. 3.8 Summary of tooth formation. (Reproduced from Nanci (2008) with the permission of the publisher)

forms posteriorly beneath the lining of the epithelium of the oral mucosa into the ectomesenchyme. Albeit at different times, permanent molars form in essentially the same manner of the deciduous teeth. While primary dentition takes place between the 6th and 8th weeks of embryonic development, permanent dentition occurs between the 20th week in utero and 10th month after birth, and the permanent first molars between the 20th week in utero and the third molar in the fifth year of life. In conclusion, the development of a tooth can be summarized in Fig. 3.8 (Nanci, 2008).

Tooth development, a complex interplay of cells, signals, and genes, serves as a model for understanding broader biological principles in development, genetics, and cell behavior. By comparing tooth formation across species, we gain insights into evolutionary adaptations. Additionally, a deeper knowledge of this process may pave the way for innovative regenerative therapies, as scientists investigate methods to stimulate stem cells for tooth repair and replacement. Overall, research in tooth development holds an immense promise for both fundamental scientific discovery and practical applications, advancing our understanding of biology, evolution, and ultimately human health.

As we have elaborated on the process of tooth development, it is applicable to examine the advancements in bioengineered tooth technology.

3.6 Bioengineered Tooth: Is It Possible?

The development of a fully functional tooth represents the highpoint of achievement in the field of tooth engineering. The history of efforts to regenerate or engineer new teeth can be compiled using strategies such as embryonic tooth bud transplantation, developmental signaling cascade manipulation, and postnatal dental stem cell-based approaches (Kamarehei & Saleh, 2024; Morsczeck & Reichert, 2018; Yelick & Sharpe, 2019; Yildirim et al., 2011; Zhang & Yelick, 2021). While Table 3.1 shows cell sources that have been used for tooth regeneration, Fig. 3.9 shows the timeline that characterizing embryonic and adult dental stem cells for tooth regeneration.

Regeneration of teeth can be broadly divided into several areas as listed below (Yildirim et al., 2011):

Table 3.1 Cell sources that have been used for tooth regeneration. (Modified from Yildirim et al., 2011)

Cell source	Species	Age	Scaffold	Method	References
Embryonic tooth bud cells	Mice	E14,5		Implantation into adult mouse	Ikeda et al. (2009)
Embryonic tooth bud cells	Mice	E14,5	Acid-soluble collagen	Transplanted into the subrenal capsule of an 8-week-old mice	Mantesso and Sharpe (2009) and Modino and Sharpe (2005)
Adult tooth bud cells	Porcine	1,5 months	Gelatin-chondroitin-hyaluronan-tri-copolymer	Autotransplantation into swine's original alveolar socket	Kuo et al. (2008)
Adult tooth bud cells and bone marrow osteoprogenitors	Porcine	6 months	PGA/PLGA	Implanted in the omenta of adult rats	Young et al. (2002, 2005)
Adult dental pulp cells	Human	18–20 years	HA/TCP	Implantation to incisor tooth extraction sockets in minipigs	Sonoyama et al. (2006)
Non-dental mesenchymal cells and + embryonic endothelium	Mice	Non-dental mesenchyme cells: 6–9 weeks embryonic endothelium: E10	Membrane filters	In vitro incubation or mice kidney implantation	Ohazama et al. (2004)
Adult gingival epithelial cells	Human	Adult human embryonic mesenchyme: E14,5		Transplantation to kidney capsules of adult SCID mice	Angelova Volponi et al. (2013)

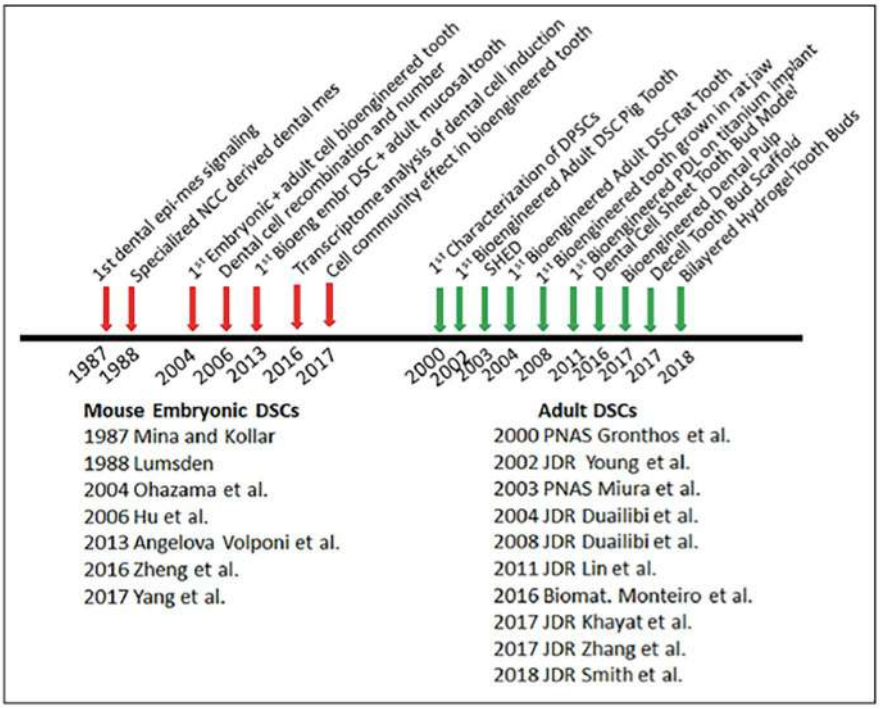

Fig. 3.9 Timeline: characterizing embryonic and adult dental stem cells for tooth regeneration. DPSC, dental pulp stem cell; DSC, dental stem cell; NCC, NC cell; PDL, periodontal ligament; SHED, stem cells from human-exfoliated deciduous teeth. (Reproduced from Yelick and Sharpe (2019) with the permission of the publisher)

- Regeneration or de novo formation of the entire, anatomically correct teeth.
- Regeneration of the dental pulp.
- Regeneration of the dentin based on biological approaches and potentially as biological fillers that may replace current synthetic materials for restorative dentistry.
- Regeneration of the cementum as a part of periodontium regeneration or for loss of the cementum and/or dentin resulting from trauma or orthodontic tooth movement.
- Regeneration of the periodontium including the cementum, periodontal ligament, and alveolar bone.
- Regeneration or synthesis of enamel-like structures that may be used as biological substitute for lost enamel (for detailed review, please refer to Yildirim et al. (2011).

If we focus solely on whole bioengineered teeth, since the landmark study of Ohazama et al. (Ohazama et al., 2004) and Ikeda et al. (Ikeda et al., 2009), the overall concept of a bioengineered tooth has just been proven in principle. Research has

demonstrated that combining adult and embryonic cells from mice and humans can successfully generate tooth primordia in vitro (Duailibi et al., 2004; Hu et al., 2006; Lumsden, 1988; Mantesso & Sharpe, 2009; Mina & Kollar, 1987; Modino & Sharpe, 2005; Ohazama et al., 2004; Young et al., 2002, 2005). Moreover, surgical transplantation of these constructs into the oral cavity has resulted in the development of fully functional, erupted teeth (Angelova Volponi et al., 2013; Duailibi et al., 2011; Ikeda et al., 2009; Kuo et al., 2008; Nakao et al., 2007). With recent advances in maintaining the inductive capacity of cultured embryonic dental cells, alongside the successful manipulation of adult cells to form bioengineered dental tissues, the prospect of clinical therapies for creating bioengineered human teeth may be closer than previously thought (Yelick & Sharpe, 2019; Zhang & Yelick, 2021).

In our research, we co-cultured ameloblast-like cells (LS8 cells) and rat dental epithelial-derived ameloblast-lineage clones (SRE-G5) with odontoblast (7F2) and osteoblast (T4-4) cell lines to induce epithelial-mesenchymal interaction in vitro (Fig. 3.10). We observed calcification at the end of the first week (Fig. 3.10 I A–B),

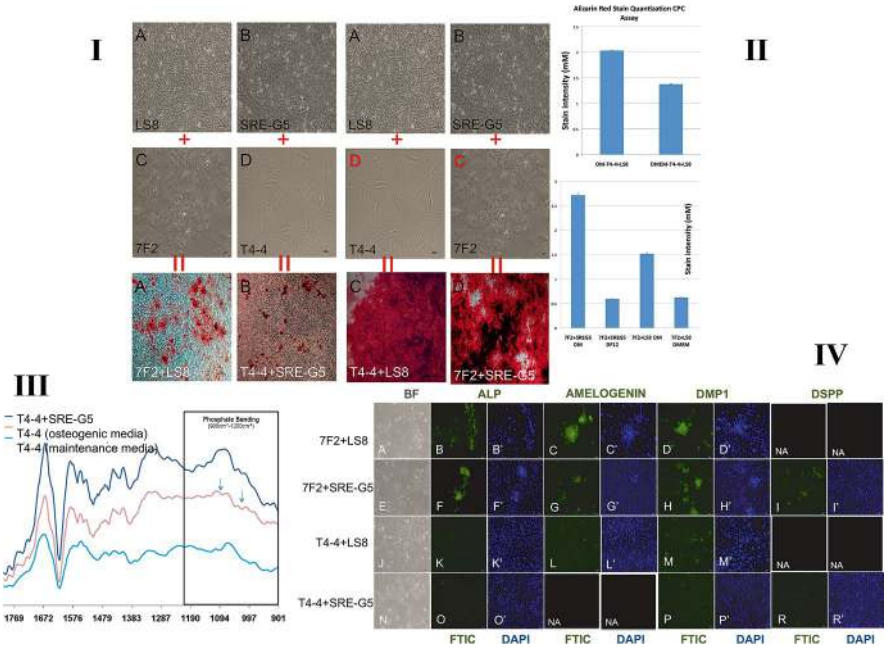

Fig. 3.10 Co-cultured ameloblast-like cells (LS8 cells) and rat dental epithelial-derived ameloblast-lineage clones (SRE-G5) with odontoblast (7F2) and osteoblast (T4-4) cell lines to induce epithelial-mesenchymal interaction in vitro. Calcification was observed at the end of the first week (I A–B), which significantly increased by the end of the fourth week (I C–D) as quantified by Alizarin Red-Staining CPC assay (II) and FITR analysis (III). There was an increase in amelogenesis and odontogenesis markers (IV)

which significantly increased by the end of the fourth week (Fig. 3.10 I C–D) as quantified by Alizarin Red-Staining CPC (Fig. 3.10 II) and FITR assays (Fig. 3.10 III).

Not only did we observe an increase in amelogenesis markers but also in odontogenesis markers (Fig. 3.10 IV). This pilot study is valuable in demonstrating that not only cells capable of initiating epithelial-mesenchymal interactions during embryogenesis but also those with amelogenic and dentinogenic potential in adulthood can participate in these interactions (Yildirim et al., 2010). Although our research is ongoing, it is important to note that these experiments focused solely on detecting dentinogenic effects.

3.6.1 Challenges for Whole-Tooth Tissue Engineering

Although bioengineering has made significant strides, several obstacles remain to be overcome in the development of a fully functional tooth, as detailed below:

- Embryonic tooth bud cells are not accessible as an autologous cell source for tooth regeneration in humans.
- Allogeneic (human) embryonic tooth bud cells are associated with ethic issues and limited availability. Xenogenic embryonic tooth bud cells may lead to dysmorphogenesis of regenerated teeth, even if it is applicable to humans.
- Adult stem/progenitor cells from the third molar or extracted teeth, per current practice, will need to be expanded ex vivo, manipulated and then transplanted into the patient, leading to unbearable cost, potential pathogen contamination, and tumorigenesis of long-term manipulated cells.
- Biomaterial scaffolds are likely essential for tooth regeneration. A tooth is not only a biological organ but also a structural material that withstand mechanical forces in mastication.
- Ideal scaffolds for tooth regeneration:
  - Should allow functionality of multiple cell types including odontoblasts, cementoblasts, pulp fibroblasts, vascular cells and/or neural endings, and potentially ameloblasts.
  - Must be clinically viable, i.e., easy to handle up to the point of a turn-key approach, can be readily sterilized and with a reasonably long shelf life.
  - Should be biocompatible and non-toxic and may need to undergo biologically safe degradation.
- Either native or synthetic polymers, or a hybrid, are valid choices as scaffolding materials for tooth regeneration.
- If biomaterial scaffolds are sufficient to recruit cells for tooth regeneration, signaling molecules are not needed.
- There is a need to determine the minimally needed signaling molecule(s) that is necessary for regeneration of tooth structures (Yildirim et al., 2011).

Besides:

- A major objective in bioengineering is to develop teeth that perfectly replicate the size and shape of the original tooth, a capability that is both highly sought after and essential for the field's advancement.
- The challenge of replicating the exact size and shape of a tooth is intertwined with the need for adequate vascularization and innervation. For bioengineered teeth to reach full size and function, a robust blood supply and nerve network are essential for growth and survival.
- Additionally, a critical factor is determining the optimal approach for regenerating teeth efficiently, ensuring they are both highly functional and seamlessly integrated with surrounding tissues like the alveolar jaw bone and periodontal ligament.
- A crucial factor for both natural and bioengineered teeth is maintaining optimal oral health, which includes fostering a balanced oral microbiome and preserving the natural protective mechanisms of saliva.
- Furthermore, the anticipated cost of bioengineered tooth therapy for humans is a significant factor that warrants careful consideration (Yelick & Sharpe, 2019).

The damaged or lost structure of teeth caused by caries or trauma can be treated clinically by replacement with an artificial material (fillings), and the outcome is usually satisfactory. Yet, tooth loss is the most common organ failure. The complex biological structure of the tooth organ including the enamel, dentin, cementum, and pulp create obstacles for any ambitious dream of the substitution of artificial material with a bio-tooth. Although the focus of the most regenerative studies is toward achieving a means to repeat evolutionary signals secondarily and initiate development secondarily, lack of understanding of the nature and interactions of tooth creating cells blocks this goal. Efforts to discover possible lineage-specific propensities within one group of cells are progressing more favorably. Although stem cells have been taken out of the body and manipulated ex vivo, there are many steps for successful cell-based therapies due to committed cells being unable to orchestrate the regeneration of complex tooth structures. To summarize, while cell transplantation and nanotechnology has shown promising results in tooth regeneration, further development of more efficient and feasible approaches is crucial for transitioning these techniques into clinical practice.

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Chapter 4
Dental Pulp Is a Connective Tissue

Although dental pulp has been classified as a loose connective tissue, several unique properties such as the presence of odontoblasts, absence of histamine-releasing mast cells, tissue confinement in a hard cavity with little collateral circulation, and vascular access limited to the root apex are the features that distinguish the pulp tissue from other connective tissues (Dummett & Kopel, 2002).

4.1 Histology of the Dental Pulp

While a dental pulp displays a characteristic arrangement in the peripheral portion, in the central core of the pulp, the basic components are arranged in a manner similar to that found in other loose connective tissues (Fig. 4.1).

A single layer of odontoblasts forms a lining at the periphery of the pulp tissue. While the cell bodies of odontoblasts stay in the pulp, the long cytoplasmic processes extend into the dentinal tubules. This specific orientation of odontoblasts results in the dentin and pulp acting together as a whole organ. This is called the "dentin-pulp complex." The anatomical, developmental, and functional relationships of the dentin and pulp make the pulpal connective tissue responsive to dental injuries, even when it is not directly stimulated (Okiji, 2002).

Subjacent to the odontoblast layer, a cell-free zone or zone of Weil is seen. This area consists of a rich network of mostly unmyelinated nerve fibers, blood capillaries, and processes of fibroblasts (Fig. 4.2).

S. Yildirim, *Dental Pulp Derived Mesenchymal Stromal Cells*,
https://doi.org/10.1007/978-1-0716-4244-3_4

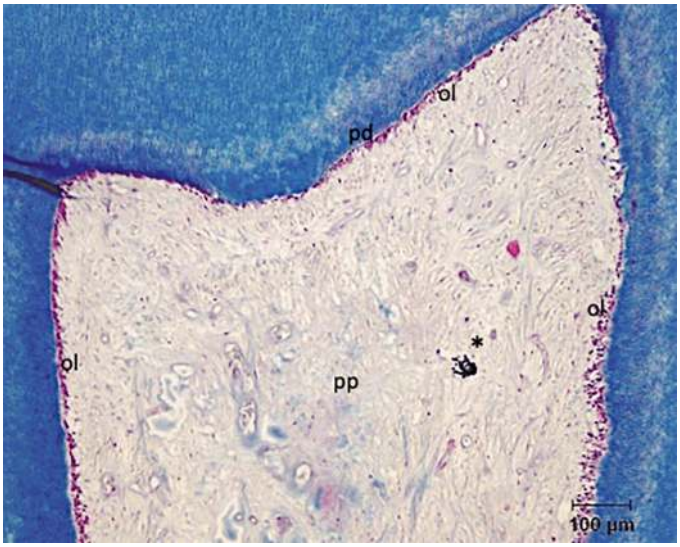

Fig. 4.1 Healthy pulp tissues seen in this histological section. *Pp* pulp proper, *ol* odontoblast layer, *pd* predentin (Masson trichrome × 10). The asterisk (*) indicates an artifact resulting from tissue processing

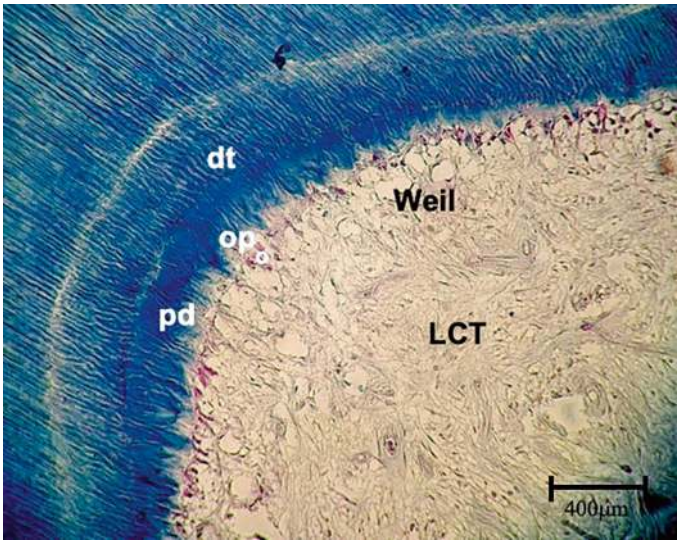

Fig. 4.2 Odontoblasts (o), odontoblastic process (op), zone of Weil (Weil), predentin (pd), dentinal tubules (dt), and loose connective tissue (LCT) of a healthy pulp histology (Masson trichrome x40)

4.2 Extracellular Matrix of the Dental Pulp

The dental pulp is classified as a loose connective tissue, which is rich in ground substance and contains relatively fewer fibers. The extracellular matrix is a major constituent of the connective tissue and includes fibrillar proteins and ground substance. While collagen and elastin constitute structural fibers, fibronectin and laminin are the main adhesive glycoproteins, whose primary function is to mediate cell-matrix interactions. The ground substance of the pulp is mainly composed of proteoglycans macromolecules, which consist of a protein core and varying numbers of glycosaminoglycan side chains. Hyaluronic acid, dermatan sulfate, and chondroitin sulfate are the most important glycosaminoglycans in the pulp (Okiji, 2002).

Collagen type I is the most abundant collagen form designated as collagen fibers in dental pulp. While the other fibrillar collagens, types III, V, VI, and XI, are seen in the pulp, non-fibrillary type II collagen does not occur. The composition of collagen types in the dentin and predentin differs considerably from that of the pulp. Since the majority of collagen molecules are produced by odontoblasts, dentin collagen might not be a combined product of odontoblasts and pulp fibroblasts (Jontell et al., 1998; Okiji, 2002). A multifunctional stromal glycoprotein fibronectin is ubiquitously distributed in the pulp. It could be localized in the pulp proper as reticular network fibrils and in the odontoblast layer. Several roles have been attributed to fibronectin including the proliferation, differentiation, and organization of odontoblasts. Elastin is always found in the pulp, as it is associated with larger blood vessels (Lesot et al., 2001; Zarrabi et al., 2011; Zhang et al., 2007).

4.3 Cells of the Dental Pulp

Physiologically, the dental pulp performs several functions, primarily induction, formation, protection, and sensation. The postnatal dental pulp contains heterogeneous cell populations in order to maintain, defend, and repair the tissue structure. The dental pulp contains various essential cell types, including odontoblasts, fibroblasts, and ectomesenchymal cells. Immune cells are also present, such as macrophages, granulocytes (including neutrophils), lymphocytes, mast cells, and plasma cells (which can present antigens). Additionally, pericytes and endothelial cells contribute to the overall cellular composition of the dental pulp (Gaje & Ceausu, 2020).

4.3.1 Odontoblasts

Although the main function of odontoblasts is dentin production, initiation of innate immune responses and suspected pain transmission are also roles attributed to them (Allard et al., 2006; Farges et al., 2009; Galler et al., 2021; Ronan et al., 2024; Yumoto et al., 2018). Fully differentiated odontoblasts are 50–60-mm-long columnar cells. Cell body and organelle distributions show polarization including one odontoblastic process containing a well-developed cytoskeleton and both exocytotic and endocytotic vesicles (Sasaki & Garant, 1996). Terminally differentiated odontoblasts secrete the first layer of the dentin (mantle dentin) and begin to move toward the center of the dental papilla. During this movement, odontoblasts leave their process behind while forming into the dentin matrix. The dentin matrix mineralizes around the process, and dentinal tubules forms. The dentin constitutes thousands of dentinal tubules that cross over the dentin from the dentinoenamel junction to the pulp. Although odontoblasts display an epithelial appearance by their alignment at the periphery of the pulp, the tight junctions between them play roles in cell polarization and differentiation processes rather than to permeability functions. However, as odontoblasts establish tight junctions between them, they create an apical domain on their surface and become fully mature. At this stage, they cease releasing matrix vesicles and start secreting various noncollagenous matrix proteins. The fully differentiated odontoblasts have a highly active secretion system (Fig. 4.3.) (Arana-Chavez & Massa, 2004; Chang et al., 2019; Couve, 1986; Couve et al., 2013; João & Arana-Chavez, 2004).

Other than the mantle dentin, the differentiated odontoblasts continue to deposit different forms of dentins: circumpulpal, intertubular, and peritubular dentins. They are all formed up until the completion of root development, and they are defined as the primary dentin. However, odontoblasts deposit the secondary dentin throughout life (Fig. 4.4). While the secondary dentin secretion rate is slower than that of primary dentin, their structures are similar (Couve, 1986; Smith et al., 1995).

As stated before, odontoblasts are highly specialized, post-mitotic NC-derived cells. They produce the collagens and proteoglycans of the organic matrix of the predentin and dentin. Odontoblasts also synthesize a variety of noncollagenous proteins, such as bone sialoprotein (BSP), dentin sialoprotein (DSP), dentin phosphoprotein (DPP), dentin sialophosphoprotein (DSPP), dentin matrix protein-1 (DMP1), phosphophoryn, osteocalcin, osteonectin, and osteopontin (OPN). DSPP, DSP, DPP, and DMP1 are considered as being dentin-specific markers; however, they can be found in several other tissues with different roles. Hence, it has been shown that DSPP is not a tooth-specific protein and that dramatically different regulatory mechanisms governing DSPP expression are involved in tooth and bone (Qin et al., 2003; Ritchie, 2018; Suzuki et al., 2012; Yamamoto et al., 2015; Yin et al., 2021). DMP1 mutations have recently been shown to cause autosomal recessive hypophosphatemic rickets, and tooth abnormalities have so far not been described in humans (Suzuki et al., 2012). DSPP affects the mineralization of the dentin more profoundly than DMP1, whereas DMP1 significantly affects bone mineralization and

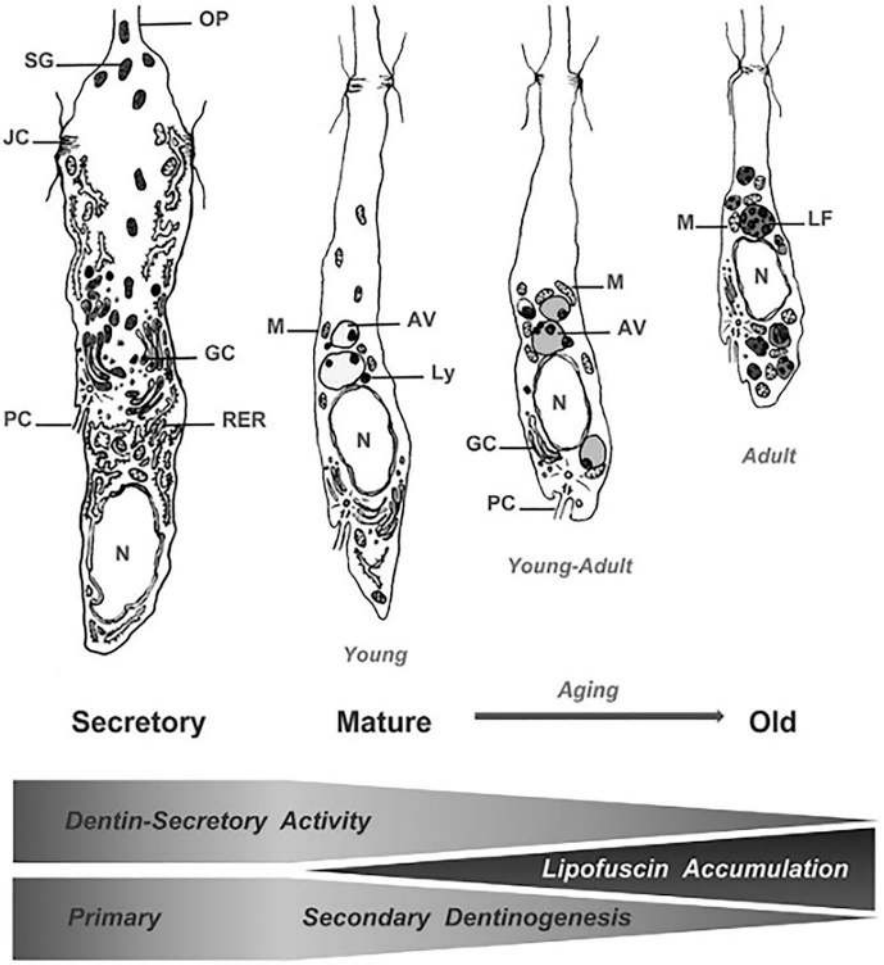

Fig. 4.3 Schematic representation of the life cycle of human odontoblasts. During tooth development, secretory odontoblasts are the "primary dentin"-forming cells. After tooth eruption at the coronal dental pulp, odontoblasts reduce their secretory machinery and acquire a mature stage characterized by the presence of autophagic vacuoles (AV). The progress of aging in mature odontoblasts involves the accumulation of lipofuscin deposits (LF). Three individual age groups are denoted in italics below their corresponding odontoblast condition. *GC* Golgi complex, *JC* junctional complexes, *Ly* lysosome, *M* mitochondria, *N* nucleus, *OP* odontoblastic process, *PC* primary cilium, *RER* rough endoplasmic reticulum, *SG* secretory granules. (Reproduced from Couve et al. (2013) with the permission of the publisher)

importantly controls serum phosphate levels. While small integrin-binding ligands, the SIBLING family of extracellular matrix proteins are not currently considered specific to dentin alone. These proteins are involved in bone and teeth mineralization and include members such as MEPE, DMP1, OPN, BSP, enamelin, DSPP, and statherin (Hao et al., 2005; Klein et al., 2022; Simon et al., 2009). CCN2, a protein

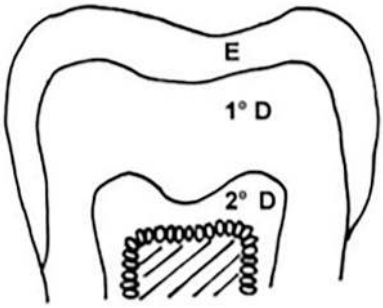

Fig. 4.4 Schematic illustration of the positions of the primary dentine (1°D) and the physiological secondary dentine (2°D) relative to enamel (E) in the human tooth. (Reproduced from (Smith et al., 1995) with the permission of the publisher)

belonging to the CCN family, has been suggested as an indicator of dentinogenesis. The latest research has shown that the levels of other CCN family members (Ccn4, Ccn5, and Ccn6) also vary during the differentiation of odontoblasts. This suggests that Ccn4, Ccn5, and Ccn6, along with Ccn2, could be used together as a comprehensive set of markers to monitor and assess dentin formation (Kawata & Kubota, 2023).

In essence, despite extensive research on the differentiation of dental pulp stem cells into odontoblasts, clear biochemical distinctions between dentin and bone remain elusive, and a specific odontoblast marker has yet to be identified.

4.3.2 Fibroblasts

Although there is detailed information about odontoblast differentiation during odontogenesis, little or no attention has been placed on the differentiation of the other cells of dental pulp. Fibroblasts are the predominant cell type in the dental pulp. They are mostly found in the cell-rich zone of the pulp, which is localized beneath the subodontoblastic layer. Their particular role is maintaining the pulp matrix. They synthesize mainly type I and type III collagens and a wide range of noncollagenous extracellular matrix components, such as proteoglycans and fibronectin. In the adult pulp, fibroblasts appear as flattened spindle-shaped cells. Fibroblasts are also included in the degradation of matrix components and thus are essential in the remodeling of the pulp tissue. Fibroblasts within a young dental pulp actively produce matrix and exhibit a distinct oval shape with a pale nucleus containing fine chromatin and abundant cytoplasm. As the demand for matrix synthesis decreases with age, these fibroblasts flatten and become spindle-shaped, displaying reduced cytoplasm and a condensed nucleus. Interestingly, when appropriately stimulated, these fibroblasts can both break down and ingest collagen. Notably, cells within the dental pulp, particularly fibroblasts, have been shown to produce inflammatory mediators such as IL-6, IL-8, and VEGF in response to bacterial components associated with caries (Gaje & Ceausu, 2020; Jontell et al., 1998; Okiji, 2002; Zanini et al., 2024).

Dental pulp fibroblasts possess unique characteristics compared to fibroblasts found in other connective tissues, notably in their expression of specific proteins like tenascin and osteonectin, as well as other extracellular matrix proteins associated with the dental pulp tissue. These vigilant cells play a multifaceted role in dental pulp biology. They produce a complete set of complement proteins, contributing to defense mechanisms, inflammation regulation, and tissue regeneration. Additionally, they generate proinflammatory molecules and express receptors that recognize pathogens, demonstrating their involvement in immune responses. They also produce neuropeptides and their receptors, influencing inflammation and wound healing within the dental pulp. Moreover, they secrete factors promoting blood vessel formation and nerve tissue maintenance, crucial for tissue repair. These cells also modulate the adaptability of nerve cells and can perceive changes in their surroundings. Please refer to a review by Álvarez-Vásquez and Castañeda-Alvarado (2022) for detailed explanations that highlight how dental pulp fibroblasts are more than mere passive cells in pulp biology and present an integrative analysis of their roles and functions (Álvarez-Vásquez & Castañeda-Alvarado, 2022).

4.3.3 Undifferentiated Mesenchymal Cells

Undifferentiated ectomesenchymal cells have been traditionally emphasized in dental literature. According to Nanci (2008), these cells are dispersed throughout the cell-rich zone and the central pulp core, frequently near blood vessels. When observed under a light microscope, undifferentiated mesenchymal cells present as large, polyhedral cells with a prominent, pale-stained nucleus located in the center (Nanci, 2008). Given the absence of known markers for undifferentiated mesenchymal cell populations, it remains unclear whether dental pulp stem cells represent a subset of these primitive cells.

4.3.4 Immunocompetent Cells

Immunocompetent cells are normal residents in the connective tissue of the pulp, and they respond to the situations that threaten tooth integrity, such as caries, tooth fracture, and cavity preparation. The human tooth is the target of a substantial number of oral bacterial agents that initiate the development of carious lesions. In the development of caries following enamel demineralization, the dentin becomes exposed to the oral environment. This enables cariogenic bacteria to cause inflammation and subsequent immune system responses in the underlying dental pulp through the diffusion of by-products into dentin tubules (Farges et al., 2009; 2015; Quispe-Salcedo & Ohshima, 2021; Schweikl et al., 2017). The dental pulp can also respond immunogenically to cavity preparation and restoration, but these immune responses are different to those caused by caries conditions (Couve et al., 2014;

Yoshiba et al., 2003). The pulp retains its defense capacity even in aged pulps (Kawagishi et al., 2006).

Inflammatory and immune system cells, such as dendritic cells, neutrophils, histiocytes/macrophages, and T/B lymphocytes, are included in the pulp. While B lymphocytes differentiate into antibody-secreting plasma cells (humoral immunity), T lymphocytes play the main part in specific immune responses (Farges et al., 2015; Quispe-Salcedo & Ohshima, 2021). Cytotoxic T lymphocytes ($CD8^+$) cause lysis of infected cells, and helper T lymphocytes ($CD4^+$) produce cytokines to orchestrate immunity. $CD8^+$ cells bind Class I major histocompatibility complex (MHC) molecules in humans very polymorphically. These are designated as human leukocyte antigens, HLA, HLA-A, and HLA-C. HLA class I molecules are expressed in almost all cells of the body. Class II MHC molecules, on the other hand, bind to $CD4^+$ molecules on T lymphocytes, and they are HLA-DR, HLA-DP, and HLA-DQ in humans. In contrast to HLA type I, type II molecules are expressed in limited types of cells, mainly on dendrites and B lymphocytes. In the intact teeth, they are distributed mainly in and around the layer of odontoblasts with dendritic profiles and are called pulpal dendritic cells (Gaudin et al., 2015; Shackelford et al., 1982; Sotirovska Ivkovska et al., 2018).

Ohshima et al. searched for HLA-DR-immunopositive cells in the human dental pulp. They found them densely distributed throughout the pulp as spindle-like or dendritic in profile. At the periphery of the pulp tissue, the HLA-DR-immunopositive cells were predominantly situated in the subodontoblastic layer, with some located in the odontoblast layer and/or predentin. These results clearly indicate that besides their roles in the defense reactions against injury, the HLA-DR-immunopositive cells in the odontoblast layer and/or predentin have some regulatory function on the odontoblasts under certain physiological conditions (Ohshima et al., 1999). Besides, Yoshiba et al. showed the immunoreactivity for HLA-DR in Schwann cells of carious lesion. Since the immunoreactivity depended upon the severity of the carious lesion, they suggested Class II-expressing Schwann cells might function as antigen-presenting cells in addition to dendritic cells (N. Yoshiba et al., 1998). Moreover, accumulating evidence shows that odontoblasts initiate immune/inflammatory events within the dental pulp in response to cariogenic bacteria (Bhol et al., 2021; Durand et al., 2006; Farges et al., 2009; Gómez-García et al., 2022; Staquet et al., 2008; Veerayutthwilai et al., 2007). Additionally, fibroblasts might recognize viruses present in the dental pulp (Glick et al., 1991; Yildirim et al., 2006; Zhong et al., 2017). However, it has been suggested that odontoblasts and pulp fibroblasts differ in their innate immune responses to oral microorganisms that invade the pulp tissue (Álvarez-Vásquez & Castañeda-Alvarado, 2022; Staquet et al., 2008).

4.4 Neuronal and Vascular Networks

The dental pulp receives a rich supply of nerve fibers originating from the trigeminal ganglion. The complex and rich neuronal and vascular networks are important for regenerative responses. With the impressive nerve fiber density of the pulp, the nerve terminals of sensory fibers store neuropeptides, such as substance P and calcitonin gene-related peptide (CGRP), neurokinin A, neuropeptide Y, and vasoactive intestinal polypeptide (Arruda-Vasconcelos et al., 2023; Caviedes-Bucheli et al., 2008; Wei et al., 2023; Zhan et al., 2024). Along with their roles in neurogenic inflammation, those peptides may modulate immune responses. It has been shown that pulpal sensory neurons have afferent pain detection and efferent (i.e., neurogenic inflammation, immunomodulatory, and healing) functions (Erdogan et al., 2024). While 70–90% of the fibers are unmyelinated, recent experiments suggest that pulpal afferents originate from fibers that are myelinated at more proximal locations (Henry et al., 2012).

4.5 Deciduous (Primary) Tooth Dental Pulp

Deciduous and permanent teeth have a similar developmental process, although the former needs a shorter time to develop. The most important difference is that the deciduous teeth undergo root resorption. The deciduous teeth start to erupt into the oral cavity by the sixth month after birth, and shedding starts around the age of 7 years. Primary molars erupt around the age of 2.5 years, and the last primary molars are shed around the age of 12 years. Hence, deciduous dentition functions for approximately 8.5 years. Their life span can be divided into periods, namely, young, mature, and old, after which resorption and shedding occur. The growth period and root maturation last about 4.75 years, and root resorption and exfoliation take about 3.5 years. Hence, permanent dentition has seven to eight times longer in which to function than the deciduous teeth (Avery, 2002). Root resorption of deciduous teeth and concurrent root formation, alveolar bone arrangement, and eruption of the successor permanent teeth in a few millimeters of space in the alveolar bone are fascinating, very well-coordinated, and yet mysterious events of the human body (Marks & Schroeder, 1996).

Deciduous and permanent teeth have similar basic dentinal structures. However, dentin thickness in the deciduous teeth is lesser than in permanent teeth, and the odontoblastic layer in the deciduous teeth is thinner than in the permanent teeth. The pulp of the deciduous teeth is proportionately larger than in the permanent teeth, and there are many accessory canals in the root pulp (Dummett & Kopel, 2002).

Clinically different pulp responses between deciduous and young permanent teeth to chemical, bacterial, or traumatic events have no counterpart histologically. Both are similar in basic histological architecture in terms of vasculature, connective tissue, and odontoblastic and subodontoblastic zones (Avery, 2002). It has been

noted that the presence of a cap-like zone of reticular and collagenous fibers in the primary coronal pulp is the only differences between the deciduous pulp tissue and the young permanent pulp tissue (Fox & Heeley, 1980). Anatomically deciduous roots have an enlarged apical foramen, in contrast to the constricted foramen of permanent roots. Despite their abundant blood supply, the deciduous teeth demonstrate exaggerated inflammatory responses, and internal and external root resorption can be seen more often than in the permanent teeth: the greater the inflammation, the more severe the resorption (Dummett & Kopel, 2002). During eruption and physiological resorption, the pulp tissue reciprocates inflammatory events by unknown mechanisms. In permanent dentition root resorption is a dental complication that occurs as a result of severe infection or traumatic events. Except the inflammation-triggered ones, root resorption is usually free of pain until the advanced stages. As discussed, deciduous teeth root resorption is a completely natural process, which allows exfoliation of the primary teeth. The observed inflammatory cell infiltration due to resorbing the apex (in deciduous teeth resorption process) is an interesting phenomenon (Fig. 4.5). It has been shown that immunocompetent cells of the deciduous tooth pulp increase in number along with the progress of physiological root resorption (Angelova et al., 2004; Durutürk et al., 2013; Simşek & Durutürk, 2005). However, the normal structure of the coronal pulp is preserved even until the tooth exfoliated (Furseth, 1968; Simşek & Durutürk, 2005), suggesting the deciduous dental pulp might have an immune-modulatory coronal pulp and resorbing apical parts. Likewise, it has been suggested that impaired accumulation of T cells within the decidua might be related to mechanisms of feto-maternal

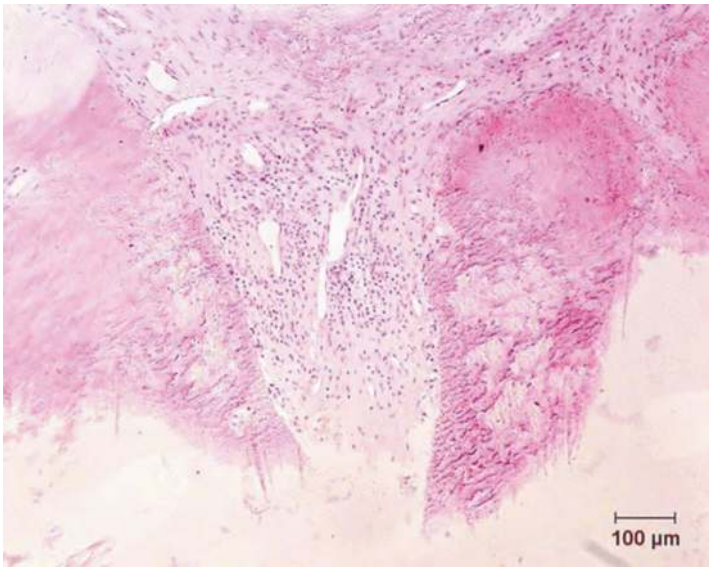

Fig. 4.5 A dense inflammatory reaction is seen throughout the apex of the resorbing deciduous toot (H&E, × 10) (unpublished data)

immune tolerance (Nancy et al., 2012). Moreover, Turan et al. (2023) proposed that during both the initial and later stages of root resorption in the deciduous teeth, apoptosis (programmed cell death) might play a role in managing the number of pulp cells, but this process may not be directly linked to the typical physiological mechanism of deciduous tooth root resorption (Turan et al., 2023). Whether such a mechanism exists, including possible epigenetic silencing of key T cells attracting inflammatory chemokine genes in deciduous dental pulp cells, and related regulation of immune tolerance of dental pulp stem cells in deciduous teeth modulating immune responses by secreting cytokines and key transcriptional factors, warrants further investigations (Yildirim et al., 2008).

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Chapter 5
Evidences for Dental Pulp-Derived Mesenchymal Stromal Stem Cells

5.1 Emergence of a New Generation of Odontoblast-Like Cells

The dentin-pulp complex has certain intrinsic healing capacities. A reparative dentin forms in response to external factors that affect the pulp, stimulating the dentinogenic progenitor cells residing within the dental pulp to initiate dentin repair (Lee et al., 2023; Rutherford et al., 1993, 1994, 1995; Rutherford & Gu, 2000; Sloan et al., 2000; Tziafas, 2019; Yildirim et al., 2011). The mechanisms for reparative dentinogenesis have been discussed extensively in the literature, and recent studies have further enriched our understanding of dental pulp and dentin regeneration (Al-Saudi, 2024; Huang, 2009; Kim et al., 2010; Li et al., 2024; Mullane et al., 2008; Rutherford & Fitzgerald, 1995; Schröder, 1985; Shah et al., 2020, 2024; Tziafas, 2019; Yu et al., 2023). However, the relationship between different characteristics of dental pulp, substantial infection or trauma of dental pulp, and intrinsic healing of dental pulp is not well understood.

Odontoblasts, as post-mitotic cells, remain in a tooth for its lifetime, secreting secondary dentin, though their synthesizing functions decrease in time (Arana-Chavez & Massa, 2004). Moreover, odontoblasts are also responsible for the secretion of a tertiary dentin in response to caries or any other mechanical or chemical problems (Fig. 5.1). Primary odontoblasts secrete dentin as secondary dentin at a very slow rate. Conditions, such as tooth decay, attrition and incidental or intentional trauma, and microleakage between restoration and dentin, stimulate the odontoblasts and their defense and/or repair responses. Several types of injurious agents can stimulate odontoblasts: bacteria and their toxins (lipopolysaccharides and other bacterial toxins), TNFα (via p38 phosphorylation of the MAPK pathway), and the liberation of cytokines and growth factors during demineralization of the dentin matrix by caries (Al Natour et al., 2023; Durand et al., 2006; Gómez-García et al., 2022; McLachlan et al., 2003; Pääkkönen et al., 2014; Paula-Silva et al., 2009).

S. Yildirim, *Dental Pulp Derived Mesenchymal Stromal Cells*,
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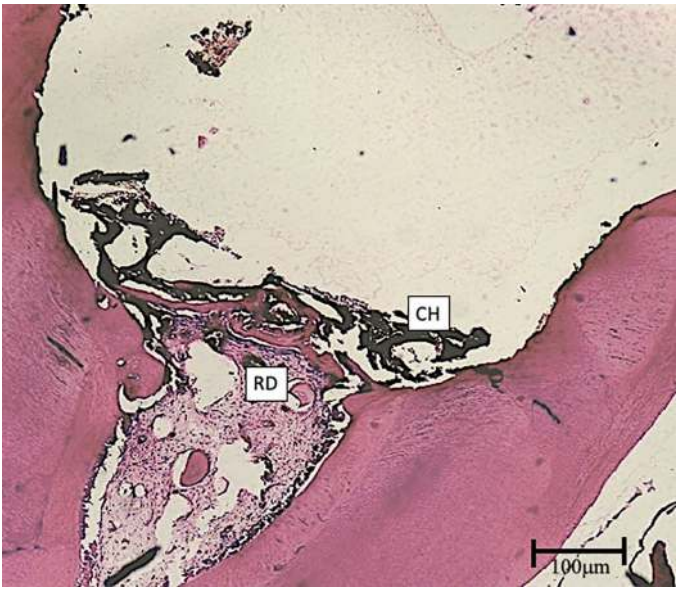

Fig. 5.1 Reparative dentin (RD) is seen in the histological section of the permanent teeth perforated and capped with calcium hydroxide (CH) after the 30th day (H&E X10) (Yildirim et al., 2011)

Once stimulated, odontoblasts enter an active state and secrete the reactionary dentin (Smith et al., 1995). However, if the nature, magnitude, or duration of the problem causes irreversible damage or the dental pulp is exposed to the oral cavity by trauma or dental procedures, primary odontoblasts die. Since the odontoblasts are post-mitotic terminally differentiated cells, they cannot proliferate to replace irreversibly injured odontoblasts. Therefore, pulpal regenerative processes take place to initiate reparative dentinogenesis, and pulp progenitor/stem cells differentiate into odontoblast-like cells, which secrete reparative dentin (Fig. 5.1). In this way, reactionary and reparative dentins are the constituents of tertiary dentin. While reactionary dentin is laid down by the surviving pre-existing odontoblasts, reparative dentin can be produced by recruited secondary odontoblast-like cells (Fig. 5.2) (Smith et al., 1995).

As we discussed in Chap. 4, initial odontoblast differentiation occurs only after a specific number of cell divisions following the initial formation of the dental lamina (Ruch et al., 1982). After this point, no new odontoblasts differentiate from the dental papilla, even when exposed to specific signals that would normally induce their formation (Tziafas & Kolokuris, 1990). Secondary or tertiary dentinogenesis on the other hand can be initiated either by the dental pulp's natural repair mechanism or through external factors. Tziafas (Tziafas, 1994) explored the fundamental developmental aspects of (tertiary) dentinogenesis, focusing on its initiation during tooth development. He reviewed clinical and experimental findings related to dentin formation, which occurs as part of pulp tissue repair

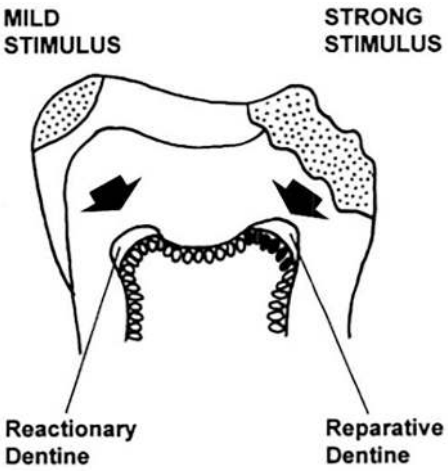

Fig. 5.2 Schematic illustration of reactionary dentine deposition in response to mild (e.g., early caries, mild abrasion/erosion) and strong (e.g., severe caries) stimuli, respectively. Note that reactionary dentine is secreted by surviving post-mitotic odontoblast cells (white), while reparative dentine is secreted by a new generation of odontoblast-like cells (black) after death of the original post-mitotic odontoblasts responsible for primary dentine secretion. (Reproduced from Smith et al. (1995) with permission of the publisher)

(with or without exposure) or due to interactions between the dentin matrix and pulp cells. Additionally, Tziafas discussed theories about the key biological steps involved in the expression of odontoblast-like cells and the subsequent initiation of tertiary dentin formation. He anticipated that the organization of the extracellular matrix within the pulp environment can direct dividing pulp cells along two different paths. In the absence of a suitable surface or under adverse conditions, these preodontoblasts create a disorganized matrix during repair (Fig. 5.3). Conversely, when attached to a surface, these cells can create a more organized matrix. The specific type of matrix formed seems to depend on the developmental stage of the cells and the nature of the surface they are attached to. When preodontoblasts interact with a dynamic surface that can bind fibronectin, they become polarized and secrete a tabular matrix. However, if undifferentiated or dedifferentiated pulp cells are attached to a favorable surface before undergoing a necessary number of cell cycles, they secrete a polar but atubular matrix (fibrodentin) without becoming polarized. This fibrodentin can then stimulate the development of new odontoblasts, similar to how basal lamina or dentinal matrix does. The success of these processes appears to rely on a healthy pulp environment, including minimal inflammation, sufficient cell density, adequate blood supply, and a specific concentration of tissue proteins that act as signaling molecules. The non-inflamed pulp tissue of young permanent teeth seems to provide such an environment (Tziafas, 1994).

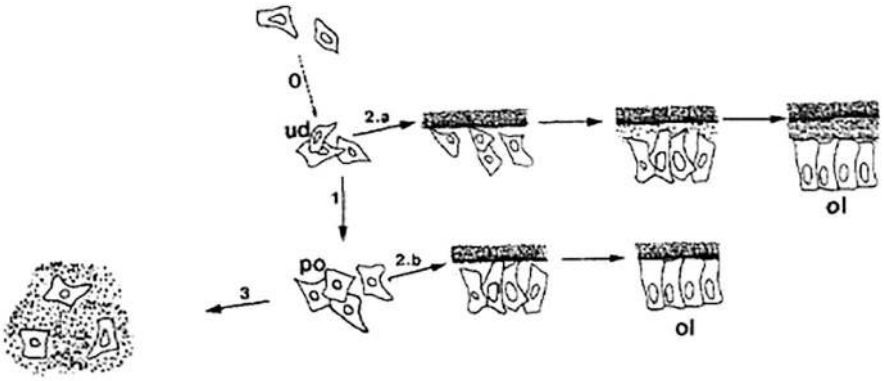

Fig. 5.3 Schematic drawing depicting a hypothetical model of mechanisms controlling secondary (tertiary) initiation of dentine histogenesis. During pulp repair, or specific inductive factors-pulp interactions, undifferentiated pulp' cells (ud) become preodontoblasts (po) when they take additional cell cycles (movement 1). Fibroblasts (and possibly endothelial cells and pericytes) dedifferentiate (movement 0) and are then changed into preodontoblasts. Further, the type of mineralized matrix synthesis seems to be dependent on the properties of the pulp environment, the differentiation status of pulp cells, and the existence of a fibronectin-rich surface to which cells can attach and become odontoblasts (ol). (Reproduced from Tziafas (1994) with the permission of the publisher)

Despite decades of research since the initial theories, fundamental questions about dentinogenesis and the origin of odontoblast-like or progenitor cells remain unanswered. While the discovery of stem cells in the dental pulp was a significant breakthrough (Gronthos et al., 2000), the nature of odontoblast-like cells remains unclear. Are they synonymous with stem cells? Definitive answers to these fundamental questions are elusive. Rather than speculating, let us focus on uncovering concrete explanations for odontoblast behavior and potential replacement mechanisms. Keeping mind that, if "ifs" and "buts" were candy and nuts, we'd all have a merry Christmas.

5.2 Origin of Progenitor Cells

The suggestion that the replacement of irreversibly injured odontoblasts by predetermined odontoblastoid cells that do not replicate their DNA after induction (Höhl, 1896) was rejected by auto-radiographic studies using tritiated thymidine (3HTdR or [^{3}H]-thymidine) (Feit et al., 1970; Fitzgerald, 1979). Metabolic incorporation of [^{3}H]-thymidine into cellular DNA is still a widely used protocol to monitor rates of DNA synthesis and cell proliferation (Hu et al., 2002). By this mean, the recruitment and induction of cell populations and DNA replication cycles before differentiation into functioning odontoblasts have been shown by several researchers (Fitzgerald et al., 1990; Tziafas & Kolokuris, 1990; Yamamura, 1985; Yamamura et al., 1980). Yamamura (1985) suggested that when a partial pulpotomy is covered

with pure calcium hydroxide, the resulting injury triggers two possible responses: either mesenchymal cells deep within the pulp revert to an undifferentiated state and then migrate to the site of injury, where they divide and eventually transform into new odontoblasts, or the existing undifferentiated mesenchymal cells are directly stimulated to differentiate into odontoblasts (Yamamura, 1985). Accordingly, Fitzgerald et al. showed that after pulp exposure in the primate teeth, there was a significant cellular influx from the pulp proper to the wound site and repair of the wound by secreting a dentin-like matrix as odontoblasts do (Fitzgerald et al., 1990). They suggested that those cells might be derived from a population of pulpal cells and perivascular tissues that reside in the deeper sites of the pulp. Additional to labeled odontoblast-like cells, they have also observed unlabeled odontoblast-like cells at the exposure site. Since "no label" indicates "no DNA replication," the authors rationally exclude the possibility of G_2-blocked, predetermined cells as a source of those unlabeled odontoblast-like cells. Instead, they suggested that those unlabeled odontoblast-like cells had replicated their DNA before the tritiated thymidine injection in those cells. Therefore, the authors concluded there is either the presence of a synchronous induction of cells that then undergo synchronous differentiation or a synchronous induction of heterogeneous population of cells (Fitzgerald et al., 1990).

These influential papers might give some ideas about the presence of dental pulp-derived mesenchymal stromal cells (or progenitors) in pulp proper, and obviously tritiated thymidine labeling was a smart tracer for their niche. Currently, perivascular cells (pericytes), undifferentiated mesenchymal/mesectodermal cells, fibroblasts, or Höhl's cells of subodontoblastic layer are proposed dental pulp progenitors. However, controversy persists regarding the origin and precise identification of these progenitor or stem cells due to inconsistencies in terminology (progenitor versus stem cells) and experimental conditions (in vivo versus in vitro).

5.3 Höhl Cell as an Odontoblast's Stem Cell: A Mere but Bold Suggestion

When regeneration in the dental pulp is considered as dentin-pulp integrity, it is seen that the majority of the literature on regeneration (repair) to date has been concerned with reparative dentin formation (Tziafas, 2019). In this scenario, the odontoblast, whose body is in the pulp and whose long-nerve cell-like process is in the dentinal tubules, is taken over by a new cell that replaces it after it dies and synthesizes a new dentin or dentin-like tissue to restore dentin-pulp integrity. In this case, the cell referred to as an odontoblast-like cell, whose identity remains elusive, is likely an odontoblast stem cell, based on current understanding.

Although Höhl cells were described in 1896 by Dr. Höhl (Höhl, 1896), they are rarely mentioned in the literature on dental pulp stem cells. However, Höhl cells are likely to be the stem cells of odontoblasts, as Höhl himself predicted that these cells

would transform into odontoblast-like cells upon the death of the original odontoblasts.

Ruch et al. (Lesot et al., 2001; Ruch et al., 1982) clearly demonstrated that the final stage of odontoblast differentiation involves an asymmetric division. After the mature preodontoblast undergoes its final cell division, only the cell adjacent to the basement membrane differentiates into an odontoblast, while the other migrates to an unidentified region or regions within the pulp. That is to say, in the asymmetric cell division of stem cells, which depends on their unique niches, the mitotic spindle of the dividing cell is positioned perpendicular to the niche surface. This special position allows only one of the two daughter cells that will be formed at the end of the division to be in contact with the stem cell niche, allowing this daughter cell to maintain its ability to renew itself (Li & Xie, 2005). Another alternative is that the self-renewal regulators are asymmetrically distributed to the daughter cells during mitosis by an internal mechanism, so that only one of the daughter cells has the ability to self-renew (F. Yu et al., 2006). As emphasized earlier (Sect. 3.3.1), the differentiation process of post-mitotic odontoblasts has many similarities with the events seen during asymmetric cell division of stem cells. In order for stem cells to renew themselves (self-renewal), with one of the two different mechanisms identified so far, they transfer the determinants related to the fate of the cell during stem cell division to only one daughter cell. Asymmetric division is a division that yields two daughter cells that are directed to a process in different directions: a stem cell divides into a mother cell identical to itself, while the other daughter cell differentiates. These different fates are manifested by the different sizes, morphologies, gene expression patterns of the daughter cells, or the cell division that will be seen later (Knoblich, 2008).

Even this brief information on the asymmetric division of stem cells shows that the position of mature preodontoblasts in relation to the basement membrane during the last cell division and the subsequent differentiation of only the daughter cell in contact with the basement membrane into odontoblast, while the other cell (Höhl cell) migrates to unknown parts of the pulp (Ruch et al., 1982), indicates that preodontoblasts divide asymmetrically according to the second scenario described above. In this case, the basement membrane acts like a niche in the final differentiation of the odontoblast. After the asymmetric division of the preodontoblast, Höhl cells "stem cells of the odontoblast" retire to the as-yet-unknown region or several different places of the pulp.

While this interpretation is theoretically very close to being correct, the lack of any marker to follow stem cells in the dental pulp leaves us helpless in collecting experimental data. In the research carried out so far, the location of stem cells has been determined by following the dividing cells in the repair processes that occur after the dentin-pulp integrity is experimentally disrupted by perforating the pulp of cells with the ability to produce dentin (Fitzgerald et al., 1990; Yamamura, 1985). When the research on this subject is evaluated collectively, it is claimed that stem cells in the dental pulp are located in the subodontoblastic area, in the rich cell layer

of the pulp, and in the central parts of the pulp, especially around the major vessels (Chen et al., 2024; Dieterle et al., 2022; Lin et al., 2011; Shi & Gronthos, 2003).

While it is established that stem cells exist within the dental pulp, the terminology "dental pulp stem cells" is problematic. While embryonic development primarily focuses on ameloblast and odontoblast differentiation, it's important to remember that the dental pulp originates from the dental papilla. However, the specific origins of the diverse cell populations within the dental pulp, each with distinct functions, remain unclear. Bearing in mind the historical development, attempts have been made to draw parallels between odontoblast differentiation during tooth development and the repair processes involving new odontoblast formation following pulp injury in adults. These newly formed odontoblasts have been variably termed odontoblastoid cells, undifferentiated mesenchymal cells, or progenitor cells. Since the discovery of stem cells in the human pulp tissue in 2000, confusion regarding nomenclature and pulp repair mechanisms has persisted. The identification of stem cells in the pulp tissue naturally led to the assumption of their central role in repair. However, while mesenchymal stem cells (or mesenchymal stromal cells) can be isolated from the dental pulp, their precise role in tissue engineering for dental repair remains under investigation. Although these cells are obtained from the dental pulp, are they stem cells of the dental pulp? The answer to the question is clearly no, because the dental pulp is a loose connective tissue that contains many different cells. This loose connective tissue is covered with a vascular-nervous network perfectly organized to perform very different functions.

While dentistry closely follows advancements in cell biology applied to medicine, it often lags behind. Although the reasons for this deserve further exploration, from a regenerative medicine perspective, the concept of stem cells within the dental pulp, a tissue with remarkable self-renewal capacity, emerged relatively early. Gronthos et al. isolated dental pulp stem cells in 2000 (Gronthos et al., 2000), just 9 years after Caplan defined mesenchymal stem cells from organ stroma in 1991 (Caplan, 1991). These cells exhibit robust proliferation and a hierarchical differentiation capacity. A PubMed search currently (August 2024) yields 4662 studies on dental pulp stem cells. These ectomesenchymal-derived stromal cells possess self-renewal potential compared to neural crest cells and are employed across diverse medical research fields (Daltoé et al., 2014; Kamarehei & Saleh, 2024; Khatami et al., 2023; Rosaian et al., 2020).

Although Höhl cells are theoretically implicated in dentin repair, our understanding of the behavior of resident stem cells in pulp repair is still limited. As previously discussed, the dental pulp exhibits a distinct cell-tissue organization peripherally, while its central region resembles other connective tissues (Okiji, 2002). A single layer of odontoblasts lines the pulp's periphery, beneath which lies Weil's cell-poor layer, rich in unmyelinated nerve fibers, blood vessels, and fibroblast processes. The pulp's loose connective tissue, originating from this layer, houses fibroblasts, most immune cells, and blood vessel components. The dental pulp harbors a stem cell population capable of re-differentiating into dentin-forming cells under appropriate conditions following dentin-pulp injury. Despite advancements in the field, the

differentiation of DPSCs into a pulp-forming cell phenotype following dentin-pulp injury has not been consistently achieved.

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Chapter 6
Dental Pulp-Derived Mesenchymal Stromal Cells

Stem cell technology is developing at a rapid pace. Every piece of seemingly unrelated, floating data is being conglomerated to broaden current knowledge and move closer to the inevitable shift in paradigms of current fundamental cell biology systems. The question of what a stem cell actually is may no longer require further discussion; however there are still some differences in opinions between groups in terms of their functional roles. As we discussed in detail in the first part of the book, the term "mesenchymal stem cell" is being replaced by other terms. Although there is no consensus in the scientific community on which name best defines these cells, in this part of the book, we will use the term "dental pulp-derived mesenchymal stromal cells" (but the abbreviation "DPSC" will remain the same in order not to lose context with the literature) when discussing cells with stem cell properties in the dental pulp.

6.1 Cellular Characterization of Adult Human Dental Pulp

As we discussed the histology of the human dental pulp in the fourth chapter, we will now continue with its cellular characterization, focusing specifically on the data recently provided by scRNA-seq analysis. A dental pulp, a specialized stromal connective tissue housed within the pulp cavity, establishes a unique microenvironment that supports tooth development, repair, and ongoing nourishment. This environment is largely isolated due to the surrounding dentin, with the only connection to the rest of the body being the narrow apical foramen in the periapical region, through which blood vessels and nerves pass (Fig. 6.1). This tissue contains a wide range of cells, including pulp cells, odontoblasts, mesenchymal cells, pericytes/endothelial cells, and immune cells, but how these cells interact with each other remains unclear.

A scRNA-seq study by Yin et al. (2021) identified 20 cell clusters in the human dental pulp including neural cells, red blood cells, immune cells, endothelial cells

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https://doi.org/10.1007/978-1-0716-4244-3_6

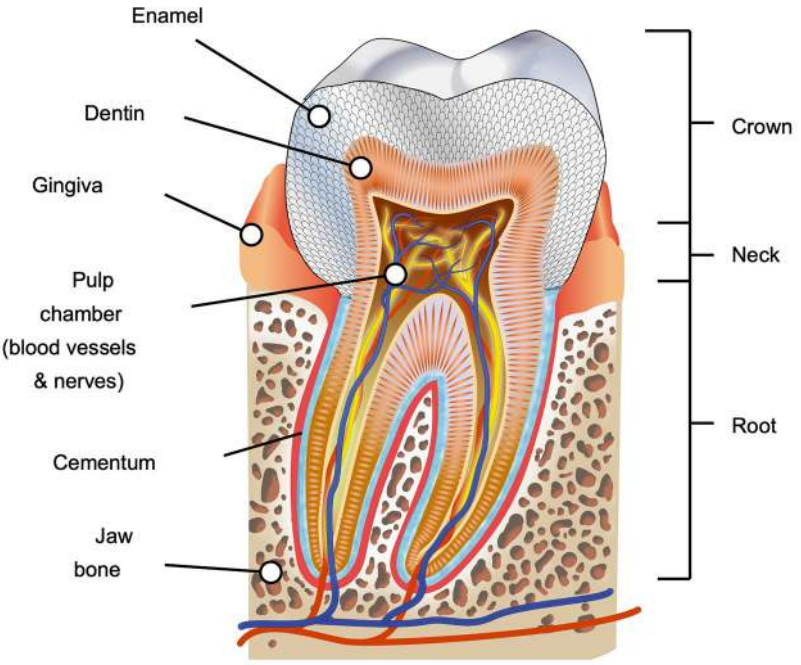

Fig. 6.1 Diagram of a healthy human molar showing the enamel, cementum, pulp, and dentin which make up the structure, as well as the surrounding tissues. (Reproduced from Human tooth diagram-en.svg from Wikimedia Commons by K. D. Schroeder, CC-BY-SA 4.0 with permission of the publisher)

(ECs), glial cells, pulp cells, and dental pulp stem cells (DPSCs). In this analysis, the researchers identified several key features of the distinct cell populations found within the dental pulp. Accordingly, the dental pulp, a complex tissue, houses diverse cell populations with distinct features. An analysis reveals varying rates of proliferation among these cells, with ECs and DPSCs being the most active. Pulp cells emerge as central communicators, establishing numerous connections with other cell types, while T cells exhibit limited interactions. The presence of immune cells, like CD8+ T cells and M1 macrophages, is notable, with T cells primarily communicating with macrophages and ECs. DPSCs, classified into three clusters based on gene expression, predominantly interact with pulp cells but also communicate with ECs, T cells, and themselves. Further analysis reveals two distinct subtypes of pulp cells, characterized by gene expression related to cell shape and differentiation. ECs are predicted to engage in close communication with pulp cells, facilitated by specific ligands and receptors. These findings shed light on the intricate cellular landscape within the dental pulp and its implications for tooth development and regeneration. Overall, this study highlights the crucial roles that pulp cells and DPSCs play in coordinating communication between cells and maintaining a balanced and stable environment within the dental pulp (Yin et al., 2021). For further details, please refer to Eldeeb et al. (2024) (Eldeeb et al., 2024) and Yin et al. (2021).

6.2 Dental Pulp-Derived Mesenchymal Stromal Cells

Adult stem cells have been proposed as undifferentiated cells found in numerous tissues throughout the body. They divide to replenish dying cells and regenerate damaged tissues (Anderson et al., 2001). To date, other than bone marrow stem cells, MSCs have been identified in a variety of tissues, such as the adipose tissue, peripheral blood, spleen, brain, synovial fluid, dermis, muscle, dental pulp, umbilical cord, skin, cornea, retina, liver, pancreas, and intestines (Prockop, 1997; Zuk et al., 2002). However, there are significant differences in their proliferation and differentiation abilities and in harvesting procedures among these MSCs (Guilak et al., 2004; Koobatian et al., 2015; Pharoun et al., 2024). One of the major obstacles in stem cell research is the difficulty in isolating them due to the lack of universally accepted markers for these cells (Lin et al., 2013).

The well-known regenerative potential of the dental pulp has indicated the existence of stem cells for decades. Finally, in 2000 Gronthos et al. isolated a population of cells that are highly proliferative and display a hierarchy for cellular differentiation and multipotentiality (Gronthos et al., 2000). Since dental pulp is derived from mesectoderm or ectomesenchyme, it has been shown that the clonogenicity, self-renewal, and proliferation capacities, expression pattern of specific markers, and multipotency of DPSC display comparative equivalence with NC cells (Aghali, 2021; Gopinathan et al., 2023; Sasaki et al., 2008; Svandova et al., 2020). DPSCs and neural crest stem cells exhibit significant similarities, including a largely overlapping gene expression profile. Both express neural crest factors (SNAIL/SNAI1, SLUG/SNAI2, TWIST1, HNK1, PAX3, NEUROGENIN2, and SOX10), neural markers (TUJ1, GFAP, and NESTIN), and pluripotency core factors (OCT4, CMYC, SOX2, KLF4, LIN28, REX1, SSEA1, STELLA, and NANOG). These shared characteristics suggest a potential connection between DPSCs and neural crest development (Luzuriaga et al., 2019; Uribe-Etxebarria et al., 2017). In general, DPSCs show the common properties of adult stem cells. According to the minimal criteria defined by the International Society of Cellular Therapy (ISSCR) for the human MSCs, these cells adhere to plastic; express CD29, CD44, CD49a-f, CD51, CD73 (SH3), CD90, CD105 (SH2), CD106, and CD166; and lack the expression of the hemopoietic surface antigens including CD11b, CD14, CD19, CD34, CD45, and CD79a and human leukocyte antigen-DR isotype (HLA-DR) and finally should give at least three differentiated lineages: osteoblastic, adipogenic, and chondroblastic (which needs to be demonstrated by staining of in vitro differentiated cell cultures) (Dominici et al., 2006). Along with expressing markers defined by the ISSCR criteria, DPSCs also express a broad spectrum of other surface markers, as detailed in Al Madhoun et al. (Al Madhoun et al., 2021). Reports from the last 30 years show many examples for the “new” stem cell lineages that completely satisfy these criteria. Despite the progress, standardized methods for evaluating the quality of MSCs derived from specific tissues have not yet been established. Ma and colleagues recently revealed that the surface molecule CD146 can be used as an

indicator of the potency of human MSC derived from dental pulp, and they have established basic criteria for evaluating this potency (Ma et al., 2021).

Liu et al. demonstrated that DPSCs express the protein programmed cell death-1 (PD-1), unlike MSCs from the bone marrow. When PD-1 expression was suppressed in DPSC, their ability to proliferate decreased significantly, and their differentiation into various cell types was accelerated (Liu et al., 2018). Single-cell RNA sequencing is a promising tool for clarifying the heterogeneity of DPSC and could help develop standardized techniques, leading to more consistent and reliable research findings (Nel et al., 2022). According to Lee et al., single-cell RNA sequencing analysis revealed that DPSC primarily consisted of osteogenic and neurogenic cell populations, exhibiting high scores for neurogenic and osteogenic differentiation potential (S. Lee et al., 2022). On the other hand, single-cell transcriptomic analysis of dental pulp and periodontal ligament stem cells revealed the lower cell proliferation capacity of PDLSC than DPSCs (Y. Yang et al., 2024). Surface markers and pre-lineages' expression profile of DPSCs has been listed comprehensively (Al Madhoun et al., 2021). Although DPSCs have been characterized by multipotent differentiation, the expression of some stem cell markers, dentin regeneration in vivo, and colony-forming ability in culture, they also contain heterogeneous populations of cells from the pulp (Calligaris et al., 2024; Gronthos et al., 2000, 2002; Nel et al., 2022; Ren et al., 2022).

6.2.1 Significant Features of DPSCs

DPSCs possess several noteworthy features that make them attractive for research and potential therapeutic applications.

6.2.1.1 High Proliferation Rates and Clonogenicity

DPSCs have a high rate of proliferation, allowing them to expand rapidly in culture, which is crucial for generating sufficient cells for therapeutic purposes. In vitro studies have shown that dental stem cells generate clonogenic cell clusters and possess high proliferation rates (Oktar et al., 2011). Moreover, stem cells from human exfoliated deciduous teeth (SHED) and DPSCs have indistinguishable fibroblastic characteristics. DPSCs are homogeneous starting from their first days in culture. They preserve plasticity during at least 25 passages (even more) while maintaining the normal karyotype and the rate of expansion characteristic of stem cells (Fig. 6.2).

DPSCs are highly proliferative. SHED show higher proliferation rates and increased population doubling time than stem cells from human permanent teeth pulp (DPSC) (Fig. 6.3).

Moreover, DPSCs are highly clonogenic. Several studies conducted in my lab have shown that DPSCs are phenotypically homogeneous from their first days in culture and highly clonogenic (Fig. 6.4).

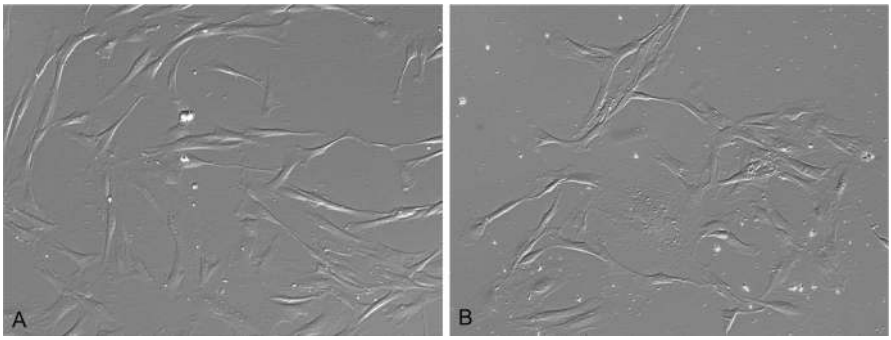

Fig. 6.2 Indistinguishable fibroblastic phenotypes of SHED (**a**) and DPSC during the first days in culture (**b**) (inverted phase-contrast microscopy, ×400)

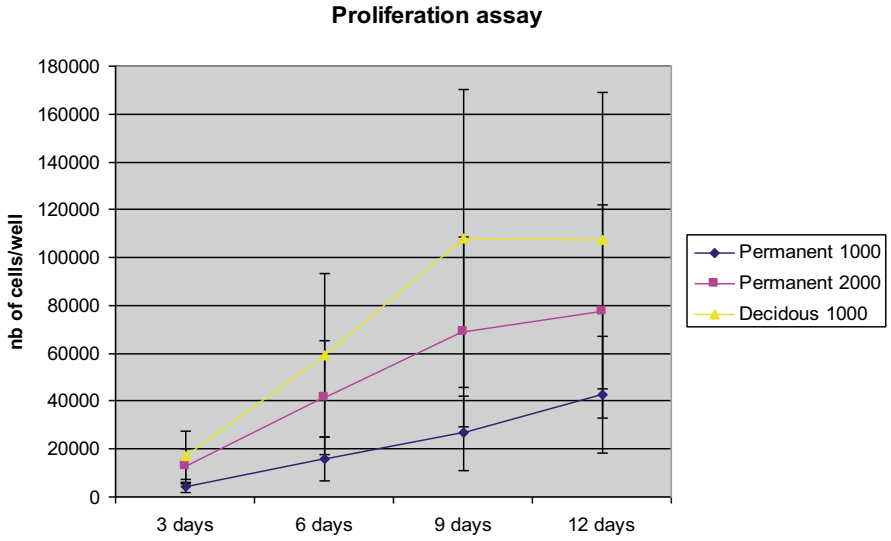

Fig. 6.3 Proliferation kinetics which measured in cells at passage 6, using two cell densities for permanent dental pulp-derived mesenchymal stromal cells (1000 cells/cm^2 and 2000 cells/cm^2) and a single-cell density (1000 cells/cm^2) for deciduous dental pulp-derived mesenchymal stromal cells

In the study, which included two different sources for MSC, we observed that human umbilical cord-derived MSC reached 90–100% confluence within 12–14 days during P0 cultures, whereas DPSC took only 8–10 days. The doubling time of human DPSCs is shorter compared to human umbilical cord-derived stromal mesenchymal cells (Oktar et al., 2011).

Using flow cytometry, cell surface marker analysis of DPSC shows consistently that they are positive for CD73, CD90, CD105 (endoglin), CD13, CD29 (integrin b1), CD44, HLA-A, HLA-B, and HLA-C. They are negative for CD45,

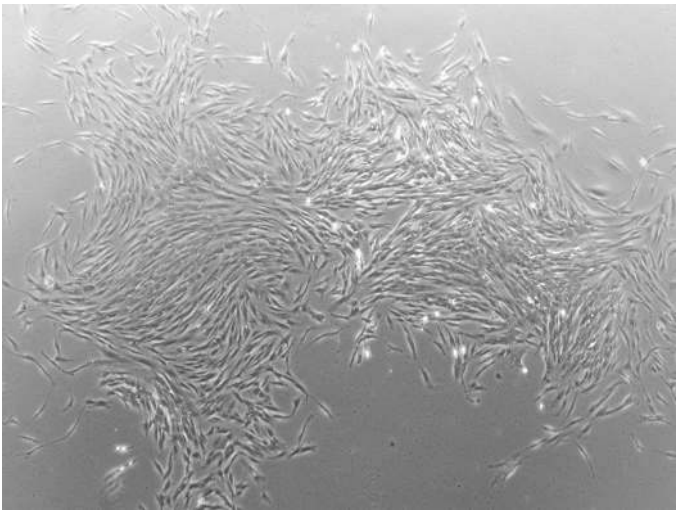

Fig. 6.4 The clonogenicity of human DPSCs becomes evident around day 11 of the very first passage

CD34, CD14, CD54, and CD133 (Oktar et al., 2011; Chen et al., 2012); thus they fulfill the criteria of MSC set by the ISSCR (Dominici et al., 2006). Besides confirming the findings described above, it has been shown that SHED and DPSC express pluripotency markers including Oct4, Nanog, c-Myc, Sox2, stage-specific embryonic antigens (SSEA-3, SSEA-4), and tumor recognition antigens (TRA-1-60 and TRA-1-81) (Fu et al., 2024; Gancheva et al., 2024; Govindasamy et al., 2010; Kerkis et al., 2006; Liu et al., 2011; Nakamura et al., 2009; Oktar et al., 2011).

Although the dental pulp is a complex and diverse tissue, the availability of "omics" data could lead to the development of more precise methods for isolating DPSCs. However, despite rapid advancements in omics research, integrating these findings into dental practice is still a challenge. Some members of the dental community are hesitantly observing the shift toward precision medicine, while others are eagerly adopting it. Continued research in dental omics is essential, but equally important is the integration of this field into the education of dental students and practitioners to ensure widespread application and benefit to patients (Kabbashi et al., 2024).

6.2.1.2 Multipotency

The ability of DPSC to differentiate into various cell types has acquired a significant interest in the field of regenerative medicine and tissue engineering. DPSCs exhibit the ability to differentiate into cells from all three germ layers: endoderm (forming organs

like the lungs, liver, pancreas, and bladder), mesoderm (producing the fat, bone, and cartilage), and ectoderm (giving rise to the skin and nerves) (Kok et al., 2022).

DPSCs are multipotent since they are capable of giving rise to various lineages of cells, when exposed to the appropriate environmental cues. Most of the DPSCs have been shown to differentiate in vitro into the desirable cells through a well-defined transcriptional cascade. Albeit the majority of the studies have focused on the ability of DPSCs to differentiate into odontoblast/osteoblast-like cells (Bakopoulou et al., 2011; Cordeiro et al., 2008; Couble et al., 2000; d'Aquino et al., 2007; Laino et al., 2005; Omagari et al., 2023; Orikasa et al., 2022; Yu et al., 2024), they can differentiate into functionally active neurons (Al-Maswary et al., 2022; Bueno et al., 2021; Kogo et al., 2020; Yoshimaru et al., 2022), mature melanocytes (Chanteloube & Debret, 2024; Paino et al., 2010; Stevens et al., 2008), smooth muscle cells (d'Aquino et al., 2007; Gandia et al., 2008; Kerkis et al., 2006), islet-like aggregates (El-Kersh et al., 2020; Govindasamy et al., 2010; Inada et al., 2022; Kuncorojakti et al., 2021), and hepatic cells (Ishkitiev et al., 2010) as well. Additionally, DPSCs have been shown to differentiate into specialized cell types such as muscle cells, heart cells, liver-like cells, pigment cells, and functional neurons. Notably, the efficiency of DPDC differentiation is significantly improved when specific conditioned media are used. Recent reviews have comprehensively covered the current understanding of DPSC differentiation into various lineages, including their angiogenic, neurogenic, odontogenic, chondrogenic, and periodontal/dental tissue regeneration potential (Al Madhoun et al., 2021; Mantesso & Nör, 2023; X. Sun et al., 2023; Vimalraj & Saravanan, 2023). In brief DPSCs can:

- Promote the proliferation and differentiation of neural cells in the hippocampus of mice (Ganapathy et al., 2019; Huang et al., 2008; Jenkner et al., 2024).
- Prevent the progression of liver fibrosis and contribute to the restoration of liver function in rats (Ikeda et al., 2008; Patil et al., 2014).
- Reduce the area of myocardial infarction, improve ventricular function, and induce the revascularization by intra-cardiac injection (Amaro-Prellezo et al., 2024; Gandia et al., 2008; Suda et al., 2022; Yamaguchi et al., 2015).
- Reconstruct the corneal epithelium in a model of total limbal stem cell deficiency (Gomes et al., 2010; Mead et al., 2015, 2017; Mohebichamkhorami et al., 2023; Syed-Picard et al., 2015).
- Form the bone when combined with platelet-rich plasma or hydroxyapatite (Abuarqoub et al., 2023; Alarcón-Apablaza et al., 2023; Gaitán-Salvatella et al., 2023; Tanikawa et al., 2020; Tomasello et al., 2017; Yamada et al., 2010).
- Repair critical-sized calvarial defects (Huang et al., 2006; Imanishi et al., 2021; Raik et al., 2022, 2023; Shiu et al., 2021; Tian et al., 2023; Yamada et al., 2010).
- Engraft and stimulate angiogenesis and vasculogenesis in models of hindlimb ischemia in mice (Iohara et al., 2008; Shekatkar et al., 2022).
- Contribute to developing the embryos of mice (Siqueira da Fonseca et al., 2009).
- Migrate, engraft, and display myogenic potential when injected into dogs (Golden Retriever) with muscular dystrophy (Kerkis et al., 2008).
- Generate tooth structures (Calabrese et al., 2024; Hu et al., 2018; Yildirim et al., 2011b).

It has been demonstrated in my lab that DPSCs obtained from a human permanent molar of a 14-year-old female patient expressed multipotency genes across multiple passages (Fig. 6.5). Similar to human permanent premolar-derived MSC, the human deciduous molar-derived MSC population also expressed Oct4 and Nanog throughout passages, while Rex-1 expression showed a random pattern (Fig. 6.6). The deciduous molar-derived cell population was found to express Notch2 and Notch3 throughout passages, but not Notch1 (Fig. 6.7).

We also determined the expression of odontogenesis-related genes, namely, alkaline phosphatase, nestin, PHEX, DSPP, DMP1, and MAP 1B, at the mRNA level in mesenchymal stromal cells derived from both deciduous, permanent dental pulps and permanent periodontal ligament using RT-PCR. The collective results of the gel analysis are shown in Fig. 6.8.

6.2.1.3 Self-Renewal

Preserving a specific pool of stem cells tailored for each tissue is vital for the functionality of most human organs and tissues (with the exception of enamel). Importantly, this preservation necessitates symmetrical cell division through the self-renewal process (Cucco et al., 2020). Human DPSCs can replicate themselves, maintaining a pool of undifferentiated cells while also generating differentiated progeny. This self-renewal capacity is essential for their long-term use in therapeutic applications. Hong et al. (2023) revealed that the OCT4A protein significantly improves the self-renewal capacity of human DPSCs by suppressing the production of a long non-coding RNA called FTX within an inflamed environment (Hong et al.,

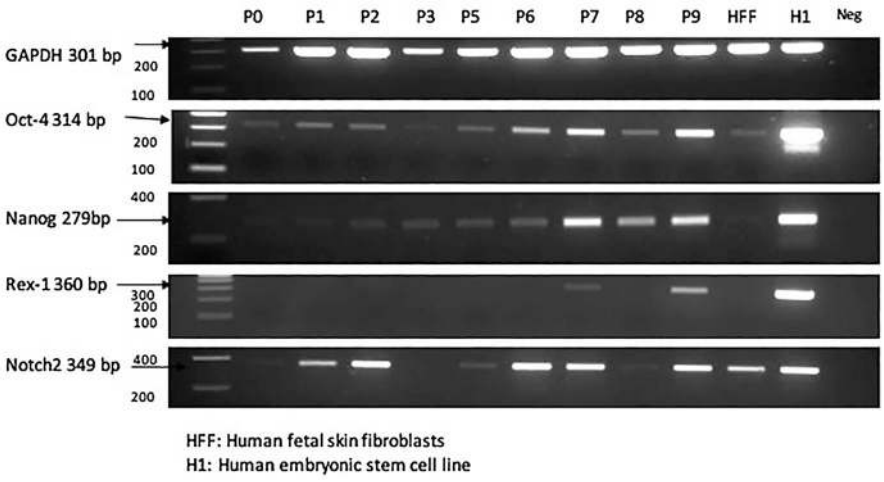

Fig. 6.5 The expression of multipotency genes in DPSC (from a permanent premolar of a 14-year-old female patient) across passages (P1-9). Those cells were observed to express Oct4, Nanog, and Notch2 in all passages. Rex-1 expression was found to emerge in later passages

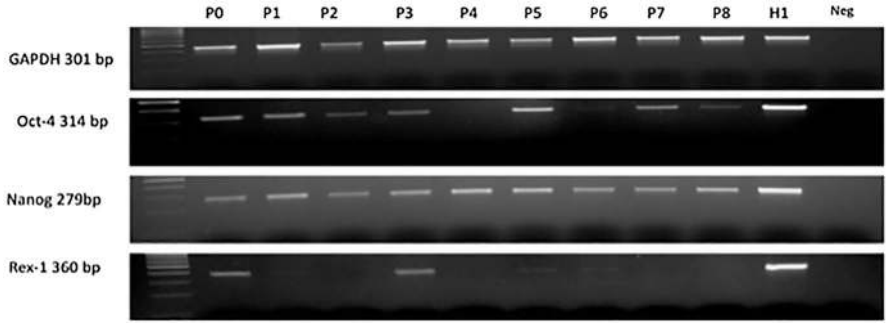

Fig. 6.6 Expression of multipotency genes in human deciduous molar pulp-derived mesenchymal stromal cells across passages. Human embryonic stem cell line (H1) mRNA was used as a positive control

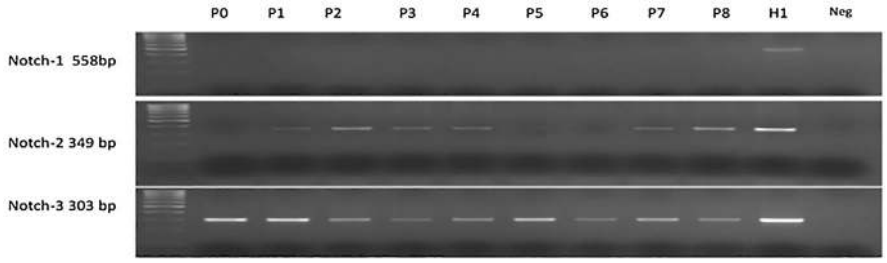

Fig. 6.7 Expression of Notch molecules in human deciduous molar pulp-derived mesenchymal stromal cells across passages. Notch1 expression was not detectable, while Notch2 and Notch3 expressions remained consistent throughout passages. Human embryonic stem cell line (H1) mRNA was used as a positive control

2023). The findings from Cucco et al. (2020) showed the importance of the SCF/c-Kit signaling pathway in promoting the self-renewal of human DPSCs. This suggests that this pathway plays a crucial role in maintaining the long-term presence of stem cells within the human dental pulp (Cucco et al., 2020).

Along with the expression of multipotency-related genes, we also investigated Notch signaling molecules and demonstrated that mesenchymal stromal cells derived from both deciduous and permanent dental pulps, as well as permanent PDL, express several members of the Notch family (Fig. 6.9). Furthermore, MSCs derived from deciduous dental pulp and permanent dental pulp, cultured on slides, were observed to react positively for SSEA-4 and TRA-1-80, two important early embryonic stem cell markers. Both cell groups also showed a strong expression for Notch2 (Fig. 6.10).

While it might be expected that these expression levels would not be entirely consistent with those observed in embryonic stem cells (Lengner et al., 2008), Pisal et al. demonstrated in their research that DPSCs do express a significant portion (17 out of 52) of pluripotency genes at levels comparable to human embryonic stem cells (Pisal et al., 2018). Moreover, a cDNA microarray system-based comparison

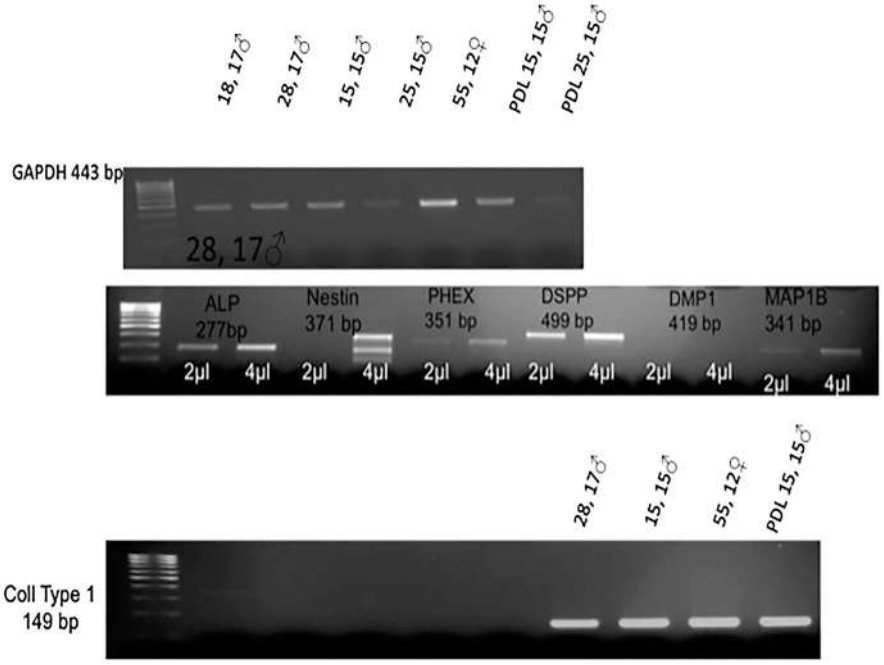

Fig. 6.8 RT-PCR results of odontogenesis-related gene expression in mesenchymal stromal cells derived deciduous tooth pulp (#55), permanent tooth pulp (#18, #28, #15, #25), and periodontal ligament cells associated with teeth 15 and 25 (PDL #15 and PDL #25)

between human DPSC and human MSC has been published recently (Yamada et al., 2010). Broadening the previous gene expression profile study for 4000 human genes in DPSC and bone marrow stroma stem cells by Shi et al. (Shi et al., 2001), Yamada et al. revealed several genes which encode the extracellular matrix components, cell adhesion molecules, growth factors, and transcription factors including Msx1 and Jun-B. According to their cluster analyses, Yamada et al. concluded that there are differences in gene expression levels for cell signaling, cell communication, and cell metabolism (Yamada et al., 2010). Using integrated miRNA and mRNA expression profiles, Chen et al. compared DPSCs and Wharton's jelly umbilical cord (Chen et al., 2012). They determined by functional network analyses that two genes (SATB2 and TNFRSF11B), which are involved in ossification, bone development, and actin cytoskeleton organization, are signatures of DPSC. They also showed upregulations of NEDD4 and EMP1, which are shown to be involved in neuroectodermal differentiation in DPSC (Chen et al., 2012). Additionally, a genetic comparison of stemness between human umbilical cord and dental pulp revealed similarities except for their differentiation potentials (Kang et al., 2016), a point on which we concur (Oktar et al., 2011). Studies have shown a close resemblance between SHED and MSC-like cells from other tissues. This resemblance is evident through the

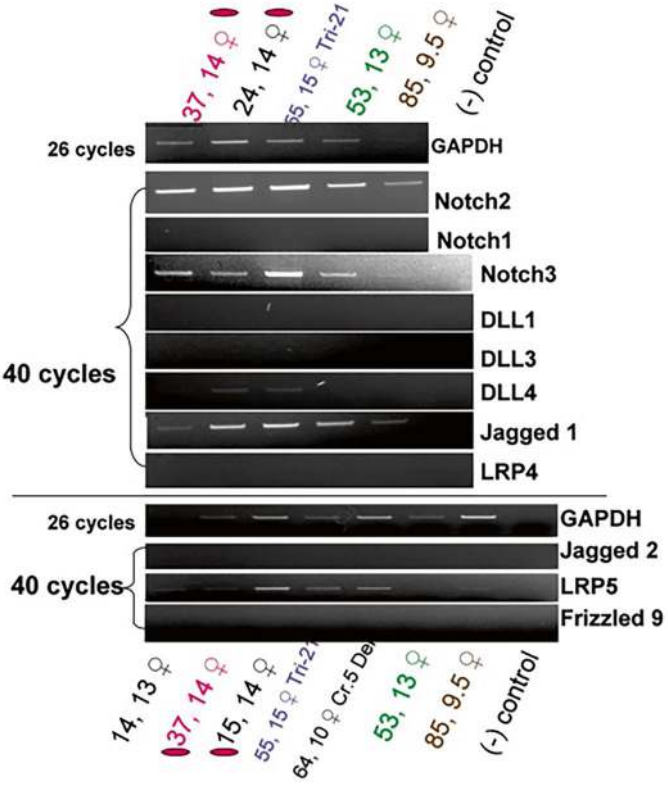

Fig. 6.9 Gel electrophoresis results of Notch signaling molecules in mesenchymal stromal cells derived from primary (deciduous) and permanent tooth pulp and permanent tooth periodontal ligament (PDL). Identical colors represent samples from the same tooth, and ellipses of the same color indicate samples from the same patient

analysis of cytosolic and nuclear proteins isolated from SHED (Pivoriūnas et al., 2010).

6.2.1.4 Immunomodulatory Properties

In addition to regenerative properties, the immunoregulatory properties of MSCs have been demonstrated both in vivo and in vitro. Because of the low expression of MHC Class II and co-stimulatory molecules, they are immunoprivileged cells. MSCs can modulate the function of immune cells with different pathways of the immune response by means of direct cell-to-cell interactions and soluble factor secretions. They display immunosuppressive effects in a number of situations. In vitro, MSCs inhibit cell proliferation of T cells, B cells, natural killer cells, and dendritic cells. The immunomodulatory effect of MSC is mediated by a nonspecific

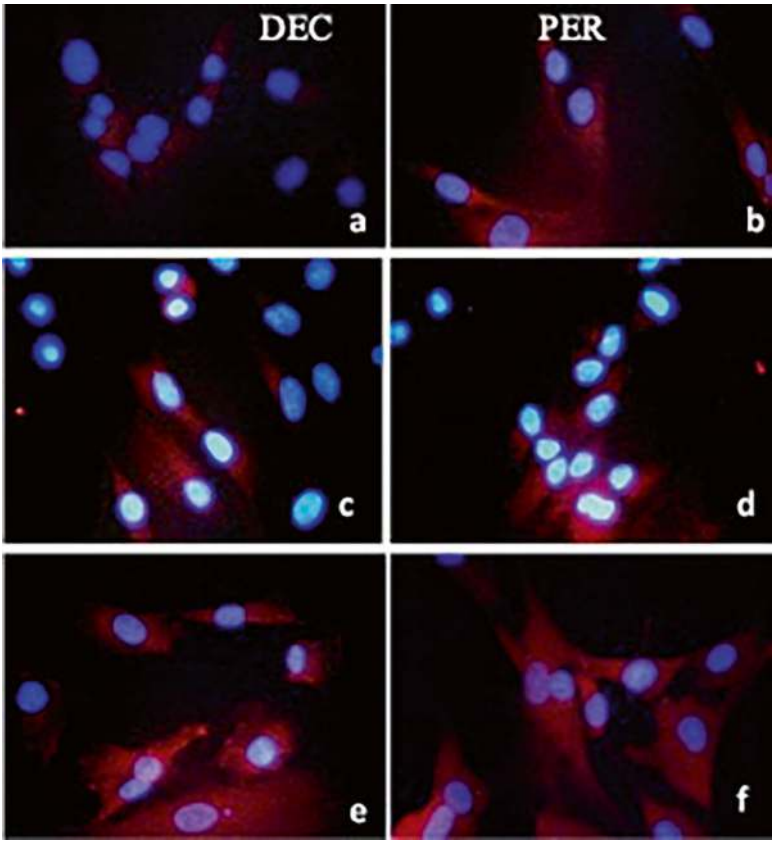

Fig. 6.10 Immunostaining of mesenchymal stromal cells derived from deciduous dental pulp (DEC) and permanent dental pulp (PER) for human embryonic stem cell markers: (a, b) SSEA-4, (c, d) TRA-1-80, and (e, f) Notch2

anti-proliferative action of these cells, and it is dependent on cell-to-cell contact or secreted soluble factors such as indoleamine 2,3-dioxygenase (IDO), prostaglandin E2, nitric oxide (NO), HLA-G, transforming growth factor (TGF)-β, interferon (IFN)-γ, and interleukin (IL)-1β (De Miguel et al., 2012; Hazrati et al., 2024; Li et al., 2023a, b; Nasef et al., 2008; Wang et al., 2022).

The interaction between DPSCs and various immune cells influences both innate and adaptive immune responses, suggesting that DPSCs possess immunomodulatory capabilities. Research advises that in cases of mild tooth injury and post-inflammatory healing, stem/progenitor cells in the tooth can help form a new dentin layer to shield the pulp from infection. These cells also exhibit the ability to regulate the immune response, either by releasing inflammatory signaling molecules or by interacting directly with immune cells. This exciting area of research warrants further investigation, as detailed in comprehensive reviews by Al Madhoun et al. (2021),

Li et al. (2014), Andrukhov et al. (2019) and Paganelli et al. (2021) (Al Madhoun et al., 2021; Andrukhov et al., 2019; Li et al., 2014; Paganelli et al., 2021).

For the first time, Pierdomenico et al. demonstrated that DPSCs display an increased immunosuppressive activity when compared to bone marrow MSC (Pierdomenico et al., 2005). Although the regulatory mechanism has not been surrogated, Huang et al. showed that the implantation of DPSC derived from rhesus monkeys into the hippocampus of mice did not cause any immune rejection (Huang et al., 2008). Later on, Tomic et al. (2011) showed DPSCs suppressed proliferation of peripheral blood mononuclear cells and that treatment with Toll-like receptor 3 and 4 agonists augmented this suppressive potential (Tomic et al., 2011).

The HLA-G non-classical MHC class I molecule was originally described in first-trimester trophoblasts at the fetal-maternal interface, and HLA-G5 is one of the major isoforms described in healthy tissues comprising trophoblast, thymus, cornea, erythroid, and endothelial precursors (Fainardi et al., 2011; Menier et al., 2010). HLA-G molecules, existing in both membrane-bound and soluble forms, have been linked to immune tolerance by modulating the activities of both innate and adaptive immune cells. These antigens can influence the killing ability of natural killer cells and CD8+ T cells, as well as the functions of CD4+ T lymphocytes and the maturation of dendritic cells (Baricordi et al., 2008). Our real-time PCR analysis revealed that HLA-G5 is expressed in the deciduous dental pulp (data not shown). Although the immunomodulatory circuits mediated by HLA-G proteins still remain to be clarified, the deciduous dental pulp may well have an intrinsic mechanism in order to manage resorption events. This relates to the hypothesis mentioned earlier that SHED might have an immunoregulatory function to modulate resorption events (see Sect. 6.5). It would be valuable to determine if, as proposed by Rizzo et al. (2011), the evaluation of sHLA-G production in IL-10-treated SHED cultures could serve as a marker of immunoregulatory function.

It is clear that there are still important issues regarding the therapeutic usage of stem cells. It has been reported that any transplanted stem cell-derived tissues, if not genetically identical to the recipient, has the potential to induce allograft rejection via “indirect” recognition, since the absence of the expression of HLA molecules cannot prevent immunological rejection because of activation of NK cells (Bryceson & Long, 2008; Taylor et al., 2011).

6.2.1.5 Paracrine Effects

DPSCs have garnered a significant attention in medical research due to their paracrine effects, which involve modulating their microenvironment through the release of bioactive molecules (Ogata et al., 2022). These therapeutic molecules can be secreted directly as the secretome or packaged within small membrane-bound vesicles called exosomes (Huber et al., 2022; Théry et al., 2002). DPSCs exert their regenerative effects through direct interaction, paracrine signaling, or autocrine mechanisms (Mattei et al., 2021; Mattei & Delle Monache, 2023). Collectively, the

molecules secreted by DPSCs, whether directly or through exosomes, contribute to tissue repair, cellular homeostasis, anti-inflammatory responses, immune modulation, and other vital functions (Li et al., 2023a; Tang et al., 2021). A proteomics study, performed to investigate the anti-inflammatory effects of molecules secreted by DPSCs under different conditions, concluded that these stem cells hold a promise as a therapeutic tool for treating inflammatory diseases (Bousnaki et al., 2022).

Studies have shown that the conditioned media (CM) from DPSCs, rich in growth factors and signaling molecules, holds a promise for treating various diseases (Chouaib et al., 2022; Ogata et al., 2022). This CM is a valuable source of the secretome, a collection of biologically active factors secreted by cells that play a crucial role in regenerative medicine (Bar et al., 2021; Kichenbrand et al., 2019). DPSCs release growth factors like VEGF, bFGF, and PDGF that promote the formation of new blood vessels. These growth factors are capable of stimulating endothelial cells to form tubular structures, a clear sign of angiogenesis (Zhou et al., 2020a, b). Shen et al. showed that DPSC-derived CM promotes angiogenesis in hindlimb ischemia (Shen et al., 2015). Furthermore, CM from cultured DPSCs has been shown to accelerate wound healing, blood vessel formation, and soft tissue regeneration in a mouse model (Yang et al., 2013). The CM from human DPSCs has been shown to influence the behavior of endothelial cells (Gharaei et al., 2018; Shekatkar et al., 2022). Additionally, these cells have demonstrated a promise in cancer treatment by effectively delivering oncolytic adenoviruses to tumors and exhibiting potent antitumor effects through their paracrine signaling (He et al., 2023). DPSCs have also shown a potential in improving glucose intolerance in diabetic mice by enhancing pancreatic β-cell function without directly differentiating into these cells (Izumoto-Akita et al., 2015).

DPSCs also produce molecules that regulate the immune system, promote the growth of blood vessels, and enhance the repair of damaged nerves. These diverse capabilities, including protection and nourishment of neurons, blood vessel formation, and immune regulation, make DPSC a promising resource for treating neurodegenerative diseases. When grown under specific conditions, DPSCs exhibit increased levels of neural stem cell markers and essential factors, suggesting their potential to aid in neural repair through both the release of beneficial molecules and their ability to transform into neural-like cells (Gugliandolo & Mazzon, 2021; Jenkner et al., 2024; Pagella et al., 2020). The secretome of DPSC contains neurotrophic factors like NDNF, NT-3, NGF, and GDNF, as well as TGF-β, all of which have shown a promise in treating neurodegenerative diseases (Dagnino et al., 2020). The CM obtained from DPSC has been proven to be more effective in promoting the survival and regeneration of trigeminal ganglion neuronal cells than other available treatments (Sultan et al., 2020). In studies with SH-SY5Y neuroblastoma cells, DPSCs were found to effectively attract these cells, stimulate the growth of neurites, and enhance immune responses related to neuronal markers (Gervois et al., 2017; Xing et al., 2023). Moreover, the findings from Miura-Yura et al. (2020) suggest that CM from stem cells from human exfoliated deciduous teeth could potentially treat diabetic polyneuropathy by stimulating the growth of neurites. The increase in

capillaries observed may also play a role in enhancing nerve function (Miura-Yura et al., 2020).

Collectively, these studies highlight the significant therapeutic potential of DPSCs and their paracrine effects in various fields, including tissue regeneration, neuroprotection, diabetes management, and cancer therapy. The secretome and exosomes derived from DPSCs are rich in trophic factors that promote tissue regeneration and proliferation. This suggests that therapies based on these factors could be highly effective for cell-free regenerative medicine, offering advantages over interventions involving cell transplantation. However, a thorough and accurate characterization of the secretome and exosomes derived from DPSCs is urgently needed (Al Madhoun et al., 2021).

6.3 Niche(s) in Dental Pulp

In healthy tissues, stem cells are found in their specific niche environment. The stem cell niche concept is coming from earlier work on the relationship between spleen colony-forming cells and hematopoietic stem cells. Schofield proposed in 1978 that the niche is a physiologically supportive microenvironment for stem cells (Schofield, 1978). Voog and Jones (2010) have defined the stem cell niche as follows:

> "Stem cell niches are discrete and dynamic functional domains that influence stem cell behavior to govern tissue homeostasis under diverse physiological (development and aging) and pathological (injury and disease) conditions. The niche must be flexible in order to coordinate stem cell behavior with homeostasis and repair; however, the plasticity of a niche may be co-opted in cancer and chronic disease.
>
> The idea that specialized environments within tissues can preserve proliferative potential and block maturation of adult stem cells was the first description of the stem cell niche hypothesis. Implicit in this model is the prediction that removal of stem cells from the niche results in loss of stem cell identity, self-renewal capacity, and the onset of differentiation. As such, the niche would provide a mechanism to precisely balance the production of stem cells and progenitor cells to maintain tissue homeostasis. Therefore, a stem cell niche is not defined solely by the presence of stem cells but also by the ability to regulate stem cell behavior." (Voog & Jones, 2010)

The niche concept nestles several factors in a multidimensional space of resources. They are interactions between stem cells and neighboring differentiated cells, adhesion molecules, extracellular matrix components, the oxygen tension, growth factors, cytokines, physiochemical nature of the environment including pH, ionic strength, and metabolites (Liu et al., 2022; Schraufstatter, 2011). Replication of a niche in vitro will allow predictable and controllable conditions for regenerative therapies. However, so far there are no available culturing conditions, which help maintain the integrity of the adult stem cells over times.

Similar to other organs, tooth compartments contain a diverse collection of stem and progenitor cells derived from embryonic stem cells. However, the specific developmental stage of most dental stem cells has not yet been determined, and

their exact function in dental development and regeneration remains unclear (Al Madhoun et al., 2021; Kaukua et al., 2014). The pulp tissue is mostly heterogeneous in nature. It would not be unreasonable to surmise that subodontoblastic and cell-rich zones of the pulp tissue may include DPSCs, since those areas are more open to attacks coming from dentinal tubules. On the other hand, those areas are also prone to destruction because of advanced caries. Hence, the safer place for stem cell residence would be pulp proper, especially at the periphery of the main arterioles, or possibly both. DPSCs may reside in the deeper pulp proper in a quiescent manner. When the "enter cell cycle" command arrives to those cells, they may divide asymmetrically. From the results of the tritiated thymidine labeling experiments, it can be assumed that, while the labeled cells found next to the wounded area are the ones that caused odontoblast differentiation to migrate to the wound site (Fitzgerald et al., 1990), the other daughter cells (possibly Höhl cells) that are the exact copies of the maternal ones may reside in this pulp proper niche for the next signal.

Alternatively, the replacement cell population in pulp healing could be located anywhere within the coronal pulp (Fitzgerald, 1979; Tziafas & Kolokuris, 1990; Yamamura, 1985; Yamamura et al., 1980). It has been shown that STRO-1$^+$ and CD146$^+$ cells are located in close proximity to blood vessels (Spath et al., 2010), and STRO-1-positive DSPCs express von Willebrand factor, CD146, α-smooth muscle actin, and a pericyte associated antigen 3G5 (Shi & Gronthos, 2003). Although the reliability of STRO-1 as a stem cell marker has been questioned recently (Lin et al., 2011), the expression of these vascular antigens has been interpreted as being that these STRO-1-type stem cells might be of stem cell origin (Dieterle et al., 2022; Shi & Gronthos, 2003).

To date, no specific markers for odontogenic neural crest or for the odontoblast lineage could be detected. However, it has been demonstrated that the Pitx2$^+$ dental epithelium and Msx1$^+$ dental mesenchyme were sufficient components for ectopic tooth germ reconstruction, although their abilities were lost postnatally (Hu et al., 2022). Yet, this research significantly enhances our understanding of dental niche cells, which appear to play a key role in controlling the interaction between epithelial and mesenchymal tissues during tooth formation (Hu et al., 2022).

The fact is that mitotic cells corresponding to stem cells are recruited in response to injury (Goldberg et al., 2006; Harichane et al., 2011; Tziafas & Kolokuris, 1990; Yamamura, 1985). The origins of these cells may be related to the primary odontoblasts, because during tooth development, the NC-derived cell population of the dental papilla gives rise to odontoblasts after an asymmetrical division. The other daughter cell, called Höhl cell, may reside in the dental pulp as a small population of stem cells within the dental pulp throughout life (see Sect. 5.3). BM is located at the cell-connective tissue interfaces of endothelial cells and Schwann cells in the mature pulp. Schwann cells are a variety of glial cells that keep peripheral nerve fibers (both myelinated and unmyelinated) alive (Lehmann & Hoke, 2010), and endothelial cells are from the endothelium, which is the thin layer of cells that lines the interior surface of blood vessels and lymphatic vessels. The endothelium forms an interface between the circulating blood and lymph in the lumen and the rest of the vessel wall (Cines et al., 1998). Since the vascular and neuronal elements in the

dental pulp are really impressive, it is rational to assume that the "niche" of the DPSCs is around the vessels and subodontoblastic area and that the dental pulp is able to specifically respond to inductive signals with the similar embryological process of BM-mediated induction for odontoblast differentiation (Ruch et al., 1982).

6.4 Epigenetics in the Dental Pulp

The term "epigenetics" was first coined by Conrad Waddington in the early 1940s (Waddington, 1942). He described it as the study of how interactions between genes and their products influence the development of an organism's observable traits (phenotype). In its original meaning, epigenetics encompassed all the molecular mechanisms that regulate gene expression and contribute to the final appearance and characteristics of an individual. According to Huang (2022), the term "epigenetics" is used in two distinct ways—molecular epigenetics and systems epigenetics—by two groups of scientists who seldom interact. Systems epigenetics, rooted in Conrad Waddington's "epigenetic landscape" concept, represents an older, broader understanding of epigenetics that existed before the rise of molecular biology. It focuses on how stable patterns of gene expression are established and maintained to create distinct cell types. In contrast, molecular epigenetics is the dominant definition today, used by molecular biologists to describe specific mechanisms like DNA methylation and histone modifications that act as "marks" to regulate gene activity (Huang, 2022). While I recommend reading this crucial article in which Huang discusses the logical inconsistencies of the molecular epigenetics paradigm, for now, let us focus on the definition and mechanisms of epigenetics in relation to DPSCs. However, it is important to bear in mind that the interpretation of these data may evolve drastically in the coming years.

Epigenetics describes mitotically heritable modifications of DNA or chromatin without altering the nucleotide sequence. DNA methylation, histone modification, histone variants, and non-coding RNAs are mechanisms of epigenetic regulation (Bird, 2002). In other words, epigenetics involves heritable changes in the structure and chemical composition of chromatin, the material that constitutes chromosomes, without modifying the underlying DNA sequence (Peixoto et al., 2020).

The microenvironmental regulation, or maintenance and differentiation of DPSCs, has recently become a subject of study. An intricate signaling network that can regulate stem cell fate in the dental pulp might be useful for understanding transcriptional and epigenetic control mechanisms of the DPSC niche. The dental pulp is a completely dynamic tissue in a constant dialogue with the environment. Teeth, and therefore dental pulp, is under the threat of bacterial and viral attacks due to caries, traumatic events, extreme heat (as a result of the hot or cold consumption), teeth grinding, dentin sensitivity, oral habits, and more (Ahn et al., 2012; dos Santos et al., 2011; Levin et al., 2020; Li et al., 2021; Ronan et al., 2024; Veire et al., 2012; Yildirim et al., 2006a, b, 2008, 2011a, b). The heterogeneous population of the dental pulp reacts by several mechanisms to those factors that might influence stem cell

quality within the niche. Hence, epigenetic events should be expected to modify the gene regulatory networks of DPSC niche(s). The development and specialization of DPSCs are tightly controlled by two mechanisms: genetic control, involving signaling pathways and transcription factors, and epigenetic modulation, encompassing modifications to DNA, histones, and non-coding RNAs (Liu et al., 2021; Zhou et al., 2020b) (refer to Table 6.1 for a summary of epigenetic enzymes influencing DPSC fate) (Liu et al., 2021).

Current literature provides a foundation for further research into the epigenetic regulation of DPSCs and its potential applications in regenerative dentistry and other therapeutic areas. By understanding how these enzymes function, researchers can develop new strategies to manipulate epigenetic modifications for therapeutic purposes. Epigenetic regulation plays a crucial role in the proliferation, differentiation, and function of dental-derived stem cells by modifying gene expression without altering the DNA sequence. DNA methylation, histone modifications, and non-coding RNAs (ncRNAs) form a complex network that independently or collectively controls gene expression. This intricate epigenetic network holds a promise for pulp and periodontal regeneration. However, despite the current focus on classical epigenetic modifications and specific sites, our understanding of epigenetic regulation in dental-derived stem cells remains limited. Future research should investigate other potential modifications like DNA 6 mA, mRNA m6A, and tRNA modifications (Y. Chen et al., 2023; Kearney et al., 2023; Pan et al., 2023). A complex communication system within the dental pulp could provide insights into how stem cells there are controlled at a genetic level. Gopinathan et al. (2023) investigated the changes in Hox chromatin organization during odontogenic lineage specification, specifically examining the epigenetic mechanisms that control HOX gene expression during the transition of neural crest cells into odontogenic progenitors. Their findings revealed a higher prevalence of repressive epigenetic marks on HOX genes in embryonic stem cells and early neural crest cells, compared to later-stage dental cells. This suggests that epigenetic regulation plays a crucial role in limiting HOX gene activity as cells differentiate into specific dental lineages. These findings highlight the importance of epigenetic regulation throughout the development of craniofacial structures from neural crest cells (Gopinathan et al., 2023). Interestingly, while mesenchymal cells are known to naturally aggregate, Svandova et al. (2020) recounted that the odontogenic and osteogenic condensations remained distinct and were separated by a loosely organized mesenchyme. Unlike the odontogenic and osteogenic ectomesenchyme derived from cranial neural crest cells, the cells of the interstitial mesenchyme do not display markers indicative of a neural crest origin (Svandova et al., 2020).

The application of epigenetic regulation to DPSCs faces challenges due to the complexity of its mechanisms. While tissue engineering using stem cells, scaffolds, and signaling molecules is feasible, epigenetic regulation often requires targeting specific modification sites, mainly achieved through virus transfection, limiting its applicability in tissue regeneration. Additionally, DPSCs influence surrounding cells through exosome secretion, highlighting the need for further research in this area (Kearney et al., 2023; Liu et al., 2021; Ye et al., 2023).

Table 6.1 "Enzymes related to epigenetic modifications of DPSCs activities" by Liu et al. (2021) with permission of the publisher (Liu et al., 2021)

Proteins and their function	Epigenetic targets	Gene manipulation of each epigenetic marker	Downstream targets	Biological process implicated
DNA methylation				
TET1, demethylation		Gene activation	*FAM20C*	(↑) proliferation, (↑) odontoblast differentiation
Histone methylation				
EHMT1, methylation	H3K9me2	Gene repression	*Runx2*	(↓) odontoblast differentiation
KMT2A, methylation	H3K4me3	Gene activation	*WNT5A*, *RUNX2*, *MSX2*, *DLX5*	(↑) odontoblast differentiation
EZH2, demethylation	H3K27me2/me3	Gene repression	Wnt/β-catenin pathway, *IL-6*, *IL-8*, *CCL2*	(↑) inflammation, (↓) odontoblast differentiation
KDM6B/JMJD3, demethylation	H3K27me3	Gene repression	*WNT5A*, *BMP2*	(↑) odontoblast differentiation
KDM5A, demethylation	H3K4me3/me2	Gene activation	*DMP1*, *DSPP*, *OSX*, *OCN*	(↓) odontoblast differentiation
Histone acetylation				
p300, acetylation	H3K9ac		*OCN*, *NANOG*, *SOX2*, *DSPP*, *Dmp1*, *Osx*	(↑) pluripotency, (↑) proliferation, (↑) odontoblast differentiation
HDAC3, deacetylation	H3K27ac		*Dmp1*, *Osx*	(↓) odontoblast differentiation
HDAC6, deacetylation				(↓) odontoblast differentiation

TET1 ten-eleven translocation 1, *EHMT1* euchromatic histone lysine methyltransferase 1, *KMT2A* lysine methyltransferase 2A, *KDM6B* lysine demethylase 6B, *KDM5A* lysine demethylase 5A, *p300* E1A binding protein p300, *HDAC3* histone deacetylase 3, *HDAC6* histone deacetylase 6, *H3K9me2* dimethylated histone H3 lysine 9, *H3K4me3/me2* tri-/dimethylated histone H3 lysine 4, *H3K27me2/me3* di-/tri-methylated histone H3 lysine 27, *H3K9ac* acetylated histone H3 lysine 9, *H3K27ac* acetylated histone H3 lysine 27, *FAM20C* FAM20C Golgi-associated secretory pathway kinase, *Runx2* RUNX family transcription factor 2, *WNT5A* Wnt family member 5A, *MSX2* Msh homeobox 2, *DLX5* distal-less homeobox 5, *CCL2* C-C motif chemokine ligand 2, *IL-6/-8* interleukin 6/8, *BMP2* bone morphogenetic protein 2, *DMP1* dentin matrix acidic phosphoprotein 1, *DSPP* dentin sialophosphoprotein, *OSX* osterix, *OCN* osteocalcin, *NANOG* Nanog homeobox, *SOX2* SRY-box transcription factor 2

Dentin-pulp regeneration relies on stem cells with the ability to proliferate and differentiate, signaling molecules that regulate cell fate, and a supportive scaffold. Epigenetic regulation, involving histone modifications, DNA methylation, and ncRNAs, plays a vital role in guiding this process. Understanding the epigenetic orchestration of DPSC fate will enhance their self-renewal, migration, and differentiation potential during pulp tissue regeneration. Promising results with histone deacetylase inhibitors in bone repair suggest a potential for dentin-pulp regeneration in vivo (Schroeder & Westendorf, 2005; J. S. Wang et al., 2021). However, more research is needed to explore the regenerative potential of DNA methyltransferase inhibitors and ncRNAs in vivo. Addressing risks associated with delivery systems, off-target effects, and neoplastic transformation is crucial before applying epigenetic strategies to optimize dentin-pulp regeneration. The use of small molecules to manipulate DPSCs' differentiation and regeneration through epigenetic mechanisms holds a great promise for developing novel approaches to functional pulp reconstruction (Chen et al., 2023; Duncan et al., 2016; Liu et al., 2021; Luo et al., 2021; Shi et al., 2023).

6.5 DPSCs from Deciduous Teeth

Various types of MSCs have been found and isolated from dental and oral tissues, such as periodontal ligament stem cells (PDLSCs), DPSCs, apical papillary stem cells (SCAP), dentinal follicle stem cells (DFPSCs), gingival stem cells (GMSCs), and deciduous teeth stem cells (SHED). Among these, DPSCs and SHED have been the most extensively studied due to their ease of access (Jenkner et al., 2024; Sugiaman et al., 2022; Zhu et al., 2021).

Since exfoliation of deciduous teeth is a physiological phenomenon and every child has 20 deciduous teeth, collecting them for isolation of adult stem cells from their pulpal remnants offers an easy, non-invasive, and ethically free method (Cakir, 2022; Kawashima, 2012). Instead, the dental pulp of permanent teeth from elderly patients has certain limitations for stem cell isolations, since the presence of subpopulations of stem cells may be greatly restricted in aged pulp cells. Because of the deleterious effects of old niches on young cells and acquired alterations in self-antigens, stem cell regenerative capacity within aged niches has been questioned (Carlson & Conboy, 2007; Farahzadi et al., 2023; Hasler & Zouali, 2005; O'Connor et al., 2009). Moreover, tight controls of regulatory networks gradually deteriorate with aging (Le Blanc & Mougiakakos, 2012; Lepperdinger, 2011; Zhao et al., 2024). Therefore, deciduous teeth and young permanent teeth might be better sources for stem cell isolation.

As a matter of fact, stem cells from human exfoliated deciduous tooth pulp have been isolated and named as SHED (*s*tem cells from *h*uman *e*xfoliated *d*eciduous teeth) in 2003 (Miura et al., 2003). They are highly proliferative postnatal stem cells capable of differentiating into odontoblasts, adipocytes, neural cells, and osteoinductive cells (Miura et al., 2003; Sugiaman et al., 2022). SHED was shown to be

capable of generating a tissue that closely resembles a human dental pulp (Cordeiro et al., 2008).

SHEDs have demonstrated superior proliferation and survival rates when compared to stem cells obtained from other dental sources (Candelise et al., 2023; Majumdar et al., 2016; Nakamura et al., 2009; Oktar et al., 2011) and other stromal sources (Huang, 2009; Oktar et al., 2011). It has been reported recently that SHED contains a higher proportion of the side population than DPSC (Wang et al., 2010a, b). Moreover, SHEDs have the potential to differentiate into functional vascular endothelial cells (Cordeiro et al., 2008; Sakai et al., 2010; Y. Zhu et al., 2013), can promote bone formation in immuno-compromised mice (Miura et al., 2003), and give better osteogenic response to retinoic acid treatment than PDLSCs (Chadipiralla et al., 2010; Winning et al., 2019).

Although some minor discrepancies regarding cell surface antigen profiles and differentiation potentials into osteogenic, adipogenic, and chondrogenic lineages have been reported so far (Govindasamy et al., 2010; G. Huang, 2009; Kerkis & Caplan, 2012; Nakamura et al., 2009; Wang et al., 2010a), the main differences between SHED and DPSC are in their proliferation rate and colony-forming properties, which are higher in SHED than DPSC (in parallel, the doubling time of SHED is shorter than DPSC) (Govindasamy et al., 2010; Nakamura et al., 2009; Oktar et al., 2011; Wang et al., 2010b). The other difference is the expression of pluripotency markers, which are again higher and more abundant in SHED. Many researchers have reported that SHED is a more primitive, pluripotent population of cells than DPSC, bone marrow-MSC, and umbilical cord stromal stem cells (Chen et al., 2012; Govindasamy et al., 2010; Kerkis et al., 2006; Nakamura et al., 2009; Yildirim, 2012). On the other hand, it has been proposed that DPSCs have a higher propensity toward neural lineage, expressing significantly higher rates than SHED (Govindasamy et al., 2010; Majumdar et al., 2016).

6.5.1 The Degree of Resorption of the Teeth Related to Stem Cell Isolation

The exfoliation of deciduous teeth is a physiologic phenomenon. Histological changes associated with the process of shedding in human beings have been well documented, and it has been shown that pulp tissue structure maintains its integrity till the late phase of exfoliation (Sahara, 2001; Sahara et al., 1996; Sasaki et al., 1989). Although there is an increase in the numbers of inflammatory cells in the active resorption area around the root(s) as well as mononuclear cells in the subodontoblastic area, regular odontoblasts still exist along the dentin walls (Fig. 6.11).

When roots are resorbed completely, odontoclasts appear in the coronal pulp (Fig. 6.12a and b), and they start to resorb predentin. In that phase coronal pulp contains many mononuclear and inflammatory cells (Fig. 6.12c). TRAP-positive mononuclear cells (presumed to be preodontoblasts) (Sahara, 2001) can be seen on

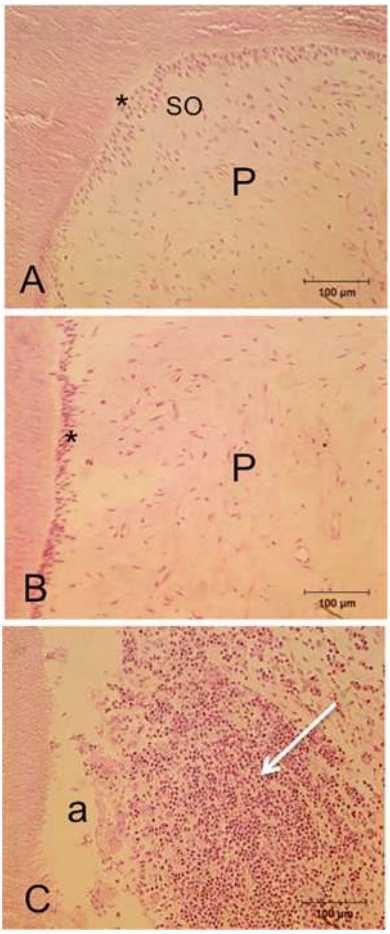

Fig. 6.11 The histological sections show healthy pulp (P) and firm layers of odontoblasts (*) and subodontoblasts in coronal (a) and cervical (b) portions, although numerous inflammatory cells invaded the resorbed root (c) (a, artificial detachment, H&E, bars 100 µm) (Yildirim & Celik, 2008)

predentin where they fused to form TRAP-positive multinucleate odontoclasts that resorbed the root (Fig. 6.13) (Yildirim & Celik, 2008).

When the resorption is completed, only minute amounts of the epithelium and periodontal ligament can retain the tooth in the dental arch, and resorbed sharp edges may cause pain during biting and eating. In the routine practice of pediatric dentistry, we rarely extract resorbed deciduous teeth, unless the patient wants relief from the pain. In that case, with little regional or only topical anesthesia, the resorbed tooth can be separated from the periodontal ligament easily and hence be extracted. Likewise, deciduous teeth are shed with little bleeding naturally, since the final shedding would occur by the tearing of the narrow tissue bridges by migrated gingival epithelium under the crown. In this final stage of resorption, there are only crowns, which their pulp chambers invaded by the oral epithelium (Sahara et al., 1993) (Fig. 6.14).

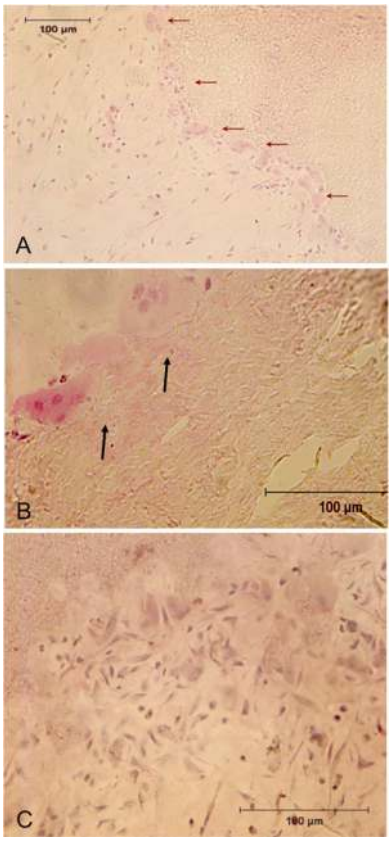

Fig. 6.12 In the final phase of resorption, odontoclasts occur in the coronal dental pulp and start to resorb predentin (arrows in **a** and **b**). In that phase coronal pulp has more heterogeneous population including many mononuclear cells (H&E, bars 100 µm) (Yildirim & Celik, 2008)

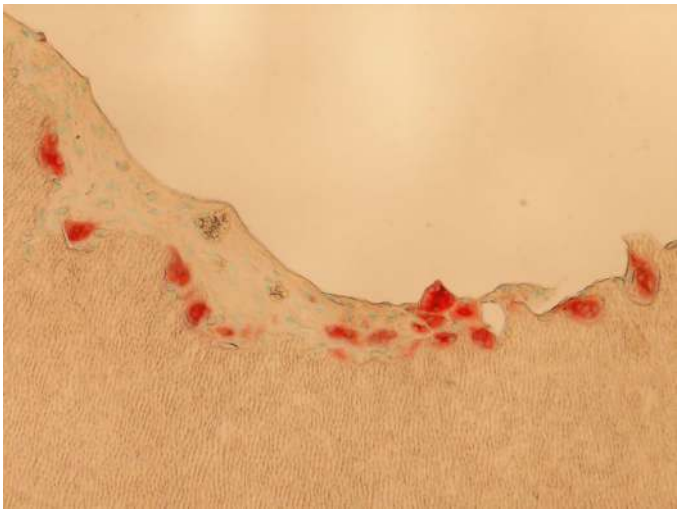

Fig. 6.13 Dark TRAP-positive multinucleate odontoclasts that reside in the lacunae on predentin (TRAP staining, ×400) (Yildirim & Celik, 2008)

Stem cell properties of deciduous dental pulp cells have been compared with the cells obtained from exfoliated and orthodontically extracted no-resorption teeth (Bernardi et al., 2011), and it has been reported that it was not possible to establish cell cultures from the teeth that did not show any visible resorption. Zhu et al. (2013) broadened the classification of resorbing teeth samples as stable, middle, and final stages and compared those samples with the non-resorbed deciduous teeth. They found that the stem cells obtained from the final resorption stage group were equal to SHED, whereas stem cells obtained from middle stage group exhibited a higher proliferation potential than those in the final stage group, yet much higher than those of the stem cells from stable resorption group (Zhu et al., 2013). My personal observation is that the pulp of a deciduous tooth very close to exfoliation, when it comes into contact with the oral epithelium, is particularly fruitful for stem cell isolation. Currently, many researchers worldwide utilize deciduous teeth for stem cell isolation.

Exciting possibilities exist despite the available pulp tissue in the exfoliated deciduous tooth being extremely small for stem cell isolation. The remnants of the pulp reside only in the pulp chamber as seen by dashed lines on the crown in Fig. 6.15, and it may occupy approximately 9–13 mm^3 in an upper incisor (if we consider the pulp chamber as being cylindrical with a radius of approximately 1 mm and a height between 3 and 4 mm). Since the pulp is a loose connective tissue, the weight of the tissue can be estimated. In order to compare stem cell properties of two different stromal tissues (human umbilical cord stroma and deciduous dental pulp), we isolated mesenchymal cells from 15-centimeter-long cords and four exfoliated deciduous teeth. At the end of P_0 (14 days), the overall yield (10^6 cells) was the same in T25 cm^2 flasks for both cell populations (Fig. 6.16) (Oktar et al., 2011).

Continuing the discussion about the increase of inflammatory molecules during resorption (Angelova et al., 2004; Simşek & Durutürk, 2005), it has been suggested that cellular influx could promote the release of cytokines acting in the activating stem cells during resorption, and the increased proliferation and mineralization potential of SHED would be a response to active resorption (Bernardi et al., 2011). It has been hypothesized that SHED may promote osteoclastogenesis, during the active resorption process via several cytokines including the RANKL/OPG duo, and this function may subside in the final stage, thereby protecting the teeth from excessive resorption (Bernardi et al., 2011; Yildirim et al., 2008; Zhu et al., 2013).

Deciduous teeth represent an ethically unproblematic source of mesenchymal stem cells due to their painless, physiological shedding at a predictable time (clinically estimable) (Cakir, 2022) and the ease with which they can be donated to researchers if not kept as a memento. As discussed in this chapter, despite the small volume of the pulp tissue in naturally exfoliated deciduous teeth, it remains a rich source of stem cells.

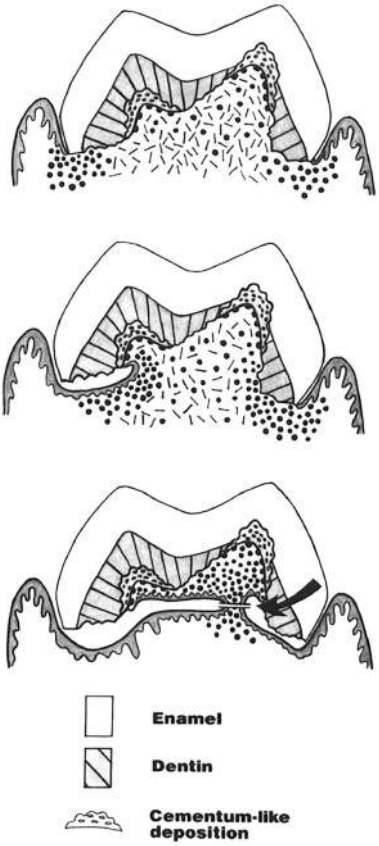

Fig. 6.14 Schematic representation of migration of the dento-gingival junction (DGJ) epithelium and gingival epithelium in the process of exfoliation of a human deciduous tooth. After the roots are completely resorbed, the DGJ epithelium gradually migrates toward the resorbed surface and finally reaches the surface of the pulp chamber wall. Accompanying this migration, the gingival epithelium also proliferates and migrates toward the resorbed regions and eventually is present under the crown. Therefore, the migrated gingival epithelium makes narrow tissue necks under the crown. The final shedding would occur by the tearing of these narrow tissue bridges (arrow) (Reproduced from Sahara et al. (1993) with the permission of the publisher)

6.6 DPSCs' Role in the Repair of the Dentin-Pulp Complex

While the possibility that DPSCs are odontoblast progenitors could be a more educated scientific prediction, considering that DPSCs play a role in the repair of the dentin-pulp complex would lead to the expectation that such a treatment is possible using these cells. As we mentioned in the tertiary dentinogenesis (Chap. 5), when primary odontoblasts, the main component of the dentin-pulp complex, are lost, DPSCs have the potential to differentiate into odontoblasts. In fact, if the ideal conditions for healing are present in the pulp (with sufficient blood supply and absence

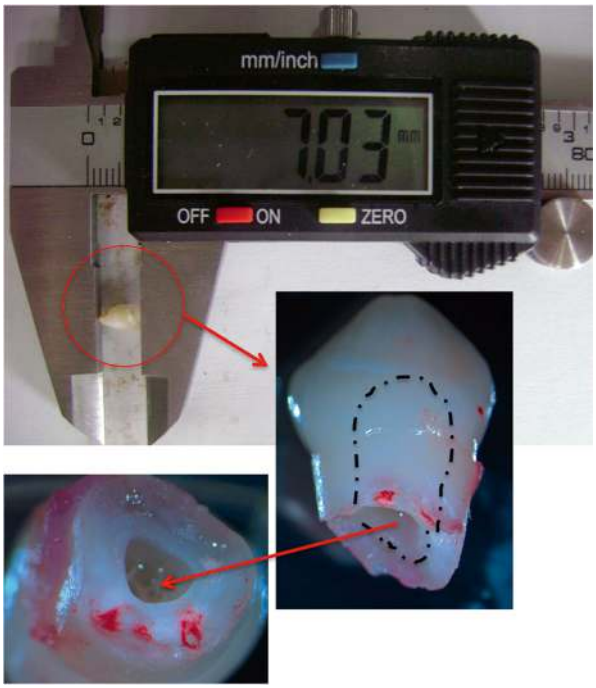

Fig. 6.15 An exfoliated deciduous tooth's height can be measured approximately as 7.00 mm including the crown. The pulp tissue resides in the coronal part. Dashed lines show the dimensions of pulp chamber

Fig. 6.16 The striking comparison of the volumes of the two loose connective tissues used for isolation of mesenchymal stem cells (Oktar et al., 2011)

of infection), such differentiation results in the physiological functioning of tertiary dentinogenesis processes.

However, there is no DPSC-based data on pulp regeneration yet. The most promising results were achieved when DPSCs were combined with biocompatible scaffolds or porous biomaterials and then transplanted. In particular, when mixed with hydroxyapatite/tricalcium phosphate (HA/TCP) or nanofibrous poly-L-lactic acid (PLLA) and transplanted subcutaneously into immunodeficient mice, DPSCs differentiated into odontoblasts and formed dentin structures with vascularized pulp-like tissue (Batouli et al., 2003; Chun et al., 2011; Cordeiro et al., 2008; Gronthos et al., 2000, 2002; J.-H. Lee et al., 2011; Miura et al., 2003; Rosa et al., 2013; Shi & Gronthos, 2003; Sun et al., 2014; Takeda et al., 2008; Wang et al., 2010a). However, neither SHEDs nor DPSCs were able to fully reconstruct the dentin-pulp complex in vivo (Al Madhoun et al., 2021; Y. Liu et al., 2021; Yoshida et al., 2020).

Moreover, three-dimensional cell culture models have been created specifically to investigate the regeneration of the dental pulp (Itoh et al., 2018; Jeong et al., 2020; F. Liu et al., 2024; X. Xu et al., 2022; S. Zhang et al., 2018). While these studies successfully fabricated organoids, they were unable to fully replicate the odontoblast layer at the pulp's edge or simulate the complete function of the dentin-pulp complex. Xu et al. suggested that in order to more accurately mimic dental pulp tissue, the fabrication of dentin-pulp organoids should incorporate other cell types or alternative strategies. Very recently, human tooth-derived organoids were established as a promising candidate for enamel and/or whole-tooth regeneration (Hemeryck et al., 2022; Hermans et al., 2024). Please refer to Liu et al. (X.-L. Li et al., 2024) and Li&Kishen (F.-C. Li & Kishen, 2023) for a detailed review on dental pulp regeneration strategies.

In conclusion, successfully regenerating functional dental pulp remains a significant challenge. Despite extensive research, we still lack the ideal combination of cells, growth factors, and scaffolds to achieve this goal. To progress further, a deeper understanding of the cellular and molecular processes involved in pulp regeneration is essential. We must also explore how cells, scaffolds, and bioactive molecules interact within the regenerating tissue.

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Chapter 7
Isolation, Cryopreservation, and Differentiation Methods of DPSC

Easy and quick isolation of the most primitive stem cell populations from available tissues is warranted for tissue engineering. The best reliable cell source for dental tissue engineering is that of autologous pulp stem/progenitor cells isolated from deciduous or permanent teeth. Easily isolated, highly expandable in vitro, and ethically uncontroversial, they are an appealing model system for numerous research applications. While the lack of universally accepted markers continues to be the major obstacle to isolating stem cells, there is also no consistent isolation and/or purification method for DPSCs (Ferrúa et al., 2017). Controversies still remain in obtaining reproducible results by the published methods especially where the differentiation protocols are concerned (Hirata et al., 2010; Iohara et al., 2006; Suchánek et al., 2010; Yu et al., 2010). Meanwhile, different isolation methods have striking impacts on the differentiation potential of adult stem cells, and the lack of consistency between the established protocols of different laboratories adds to the challenge of interpreting previously reported data (Bakopoulou et al., 2011, 2017; Bui et al., 2021; Liu et al., 2006; Wang et al., 2024).

7.1 Isolation of DPSCs

Extracted teeth by routine dental practice and exfoliated deciduous teeth by physiological resorption are human dental pulp sources. Isolation of DPSCs is quite easy and completely non-invasive: The dental crown should be disinfected briefly before extraction with 0.3% chlorhexidine and after extraction with iodine and 70% ethanol. Teeth should be placed into a transport medium, if transported to another lab for isolation procedures. Transport medium should include at least 3% (w/v) penicillin and streptomycin and 5 μg/mL amphotericin B. Since the oral

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cavity has the second highest proportion of microbiomes in humans (Jumpstart Consortium Human Microbiome Project Data Generation Working Group, 2012), all instruments should be sterilized against the risk of a microbial contamination. Permanent teeth should be cracked with a mini-hammer in order to get access to the pulp chamber and root canals. The use of a high-speed dental handpiece to section a tooth in order to access the pulp chamber and canals is both inefficient and detrimental to the preservation of pulp vitality. A more expedient and less invasive approach involves the application of a single, controlled blow from a sterilized small hammer to fracture the tooth. This technique allows for rapid access to the pulp tissue without the risk of iatrogenic injury. Dental forceps may be employed to facilitate the procedure if required. The crowns of resorbed deciduous teeth may be cracked as well, since the pulp tissue remnants reside in the pulp chamber. Once a clear visual access is established, the pulp tissue can be taken out with toothless surgical pliers.

Three primary methods are described in the literature for isolating DPSCs, namely, explant culture, enzymatic digestion, and mechanical dissociation, with the latter two sometimes used in combination (Ferrúa et al., 2017).

7.1.1 Enzyme Digestion Method

After washing with sterile phosphate-buffered saline (PBS), the pulp tissue should be cut into as small pieces as possible (~1mm^3 pieces). Tissue pieces should be washed twice with sterile PBS and placed in a solution of 2 mg/ml collagenase type I and dispase for 30–45 min at 37 °C with gentle agitation on an orbital shaker (Oktar et al., 2011). Cell suspensions can be obtained by passing the tissues through a 70-μm cell strainer. Alternatively, the homogenate should be centrifuged at 130 *g* for 5 min, and the pellet should be re-suspended in the growth medium. Then, T25 cm^2 cell culture plates can be used to culture and expand DPSC up to several passages.

There is a significant diversity in the combinations of enzymes used for cell isolation through enzymatic digestion across different studies. There are different enzymatic combinations such as 3 mg/ml collagenase type I and 4 mg/ml dispase for 30–60 min at 37 °C (G. Huang et al., 2006; Liu et al., 2006; Tirino et al., 2011), 1 mg/mL collagenase type I and 2.4 mg/ml dispase (Lee et al., 2011b), 2 mg/mL collagenase type I and dispase for 30 min at 37 °C (Yildirim et al., 2012), or 0.2% trypsin for 5 min at 37 °C (Spath et al., 2010) or 3% collagenase type I for 1 h 37 °C (Yan et al., 2011). Ferrúa et al. (2017) reviewed methodologies for DPSC isolation, highlighting the most frequently used techniques. In their scoping review of 222 studies, authors reported the distribution of enzyme types used for DPSC isolation, both with the enzymatic technique alone and in combination with mechanical techniques (Table 7.1) (Ferrúa et al., 2017).

Table 7.1 The distribution of enzyme types used for DPSC isolation, both with the enzymatic technique alone and in combination with mechanical techniques. (Reproduced from Ferrúa et al. (2017) with the permission of the publisher)

Variable	n	%
Collagenase type I and dispase[a]	111	54.4
Collagenase type I	35	17.2
Collagenase and dispase[a]	14	6.9
Collagenase[a]	9	4.4
Collagenase type I and dispase type II	8	3.9
Trypsin	5	2.5
Collagenase type I and collagenase type II	4	2.0
Collagenase type IA	2	1.0
Collagenase type II	2	1.0
Dispase type I	1	1.0
Collagenase type A	1	1.0
Collagenase type IA and dispase[a]	1	1.0
Collagenase type II and dispase type I	1	1.0
Collagenase and dispase type II[a]	1	1.0
Collagenase/DNAse	1	1.0
Collagenase type I and DNAse	1	1.0
Trypsin and collagenase	1	1.0
Collagenase blend type H	1	1.0
Collagenase type I, collagenase type II, thermolysin	1	1.0
Not described types of enzymes used	3	1.5
Total	203	100.00

[a]The type of collagenase and/or dispase was not described

7.1.2 Outgrowth (Explant) Method

The outgrowth method, while less common in laboratory studies due to its longer cell culture duration (typically 1–2 weeks), offers practical advantages such as reduced cell isolation costs and improved confidence in cell performance due to the lower operational risk (Hilkens et al., 2013). In the explant outgrowth method, cells originate from a piece of pulp tissue and are grown outward within a culture flask. This method involves cutting the pulp tissue into roughly 1 mm^3 pieces and placing them in 24-well plates. To facilitate the mechanical dissociation of dental pulp tissue into smaller fragments, a recommended approach involves the deposition of a pulp tissue into a 1.5 mL Eppendorf tube pre-filled with an appropriate culture medium. Subsequently, the tissue is subjected to rapid and precise mincing using the sharp tips of a small surgical scissor. Once the tissue pieces adhere to the bottom of the wells, they are submerged in a preferred culture medium supplemented with 10% FBS and 100 U/mL Pen/Strep. The cells are then allowed to proliferate within this environment. The plates are incubated at 37 °C in a 5% CO2 atmosphere (Fig. 7.1).

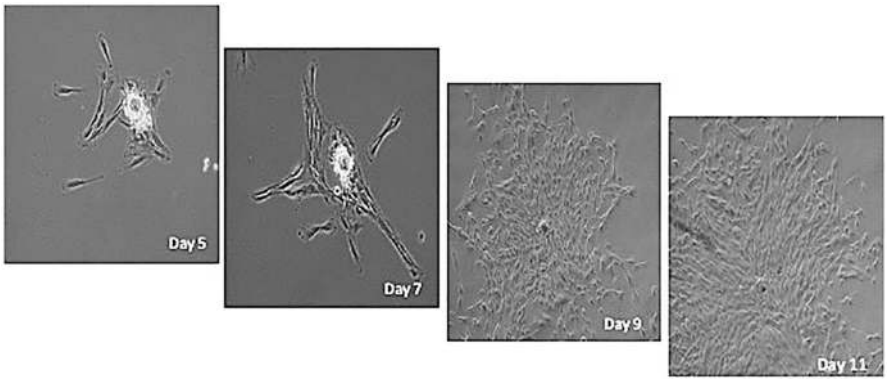

Fig. 7.1 Typical DPSC colony formed around the dental tissue explant, observed on days 5–11 of passage 1

The ideal method for isolating and culturing DPSCs should prioritize preserving their natural state and minimizing cellular damage. While enzymatic digestion may offer higher proliferation rates, concerns have been raised about the potential harm to the cell membrane or loss of stem cell properties compared to the tissue outgrowth method. According to Lampiasi (2023), the explant method allows for the observation of cell migration, as DPSCs gradually emerge from the tissue, while non-migrating cells remain inside and may migrate later (potentially reflecting DPSCs) residing in different niches (Mitsiadis et al., 2017) or undergoing apoptosis if not stem cells. Although the explant method yields a more homogenous DPSC population in subsequent waves, unattached and adherent cells that are not DPSC may also be present initially. However, these unwanted cells are eliminated over time: unattached cells are removed during media changes, and adherent non-DPSCs cannot survive or proliferate and are lost during early subcultures (Lampiasi, 2023).

Although both methods appear to give rise to pulp cells that have stem cell properties (About et al., 2000; Batouli et al., 2003; Couble et al., 2000; Gronthos et al., 2000), the effects of various isolation methods on pulp cell populations have also been questioned. One of the earliest studies by Nakashima showed that four different enzymatic separations (trypsin, collagenase with and without trypsin treatment, and a trypsin-collagenase mixture) had no effect on the morphological characteristics of the cultured pulp cells, but the trypsin pre-treatment/collagenase method gave the highest number of isolated cells. While she reported three different morphologies as spindle, stellate, and epithelial-like cells (Nakashima, 1991), Huang et al. showed compact and loose colony types after enzymatic digestion (Huang et al., 2006). Since enzymatic digestion causes all cell types to be released from the tissue, it is not surprising to observe different cells. However, only cells that have a fibroblastic appearance were able to survive after several passages because of the usage of mesenchymal cell-promoting culture media (Huang et al., 2006; Lampiasi, 2023). On the other hand, Bakopoulou et al. showed that in the enzymatically digested samples, the mineralization rate and the amount of mineralized matrix produced were higher compared to outgrowth cultures. However, the researchers used

the cell culture media which were different for the digested and outgrown cultures (Bakopoulou et al., 2011). Contrarily, Spath et al. displayed enhanced differentiation abilities with explant cultures when compared to enzymatically digested DPSC (Spath et al., 2010). However, they cultured trypsin pre-treated explants onto a Petri dish coated with fibronectin in a MegaCell complete medium. Dastgurdi et al. observed that the type of enzymatic digestion method influenced CD146 expression. Specifically, cells isolated using a collagenase/dispase combination showed higher levels of CD146 compared to those isolated with collagenase alone (Dastgurdi et al., 2018).

Additionally, Kerkis and Caplan (2012) reported that they obtained immature dental pulp stem cells (IDPSCs) from deciduous teeth by using enzymatic digestion and DMEM low glucose supplemented with 10% FBS, while the outgrowth method using DMEM/F12 supplemented with 15–20% FBS gave IDPSC (Kerkis & Caplan, 2012). While it is rational to think that SHED is heterogeneous compared to single cell-derived colonies of IDPC, wider screening gene expressions of the latter do not prove that these two isolation systems gave different cells. Moreover, Kerkis and Caplan (2012) only screened the deciduous dental pulp cells isolated via the outgrowth method and compared their data with the ones obtained by the other groups, which does not include the same set of genes (Kerkis et al., 2006; Miura et al., 2003; Pivoriūnas et al., 2010; Yamaza et al., 2010). Although favorable characteristics have been reported for DPSC obtained through the explant method (Machado et al., 2015), enzymatic digestion methods for SHED isolation have shown more promising results regarding stem cell properties compared to the outgrowth method (Bakopoulou et al., 2011).

The most significant differences for enzymatic digestion and outgrowth methods are in reported cell number and homogeneity. While the former is always higher in enzymatic digestion, tissue explants allow the expansion of fibroblastic-like cells in dental pulp cultures (Hilkens et al., 2013). To leverage the advantages of the explant technique, Wu et al. demonstrated that cell proliferation could be achieved by reusing the same explant up to four times, after the initial explant culture colony reached the ideal confluence level (80%) (Wu et al., 2024). A recent study reported that the explant culture method, using an explant outgrowth approach on basement membrane-coated flasks, consistently produced sufficient cell numbers for further expansion and experimentation (Kearney et al., 2024). In conclusion, there is a consensus that nearly all DPSC populations exhibit complete or partial overlap in the expression patterns of analyzed markers, regardless of the isolation method employed (Huang, 2009; Kerkis & Caplan, 2012).

7.1.3 The Other Isolation Methods for DPSC

Since there are no identified DPSC marker(s), there are continuing efforts for the further purification of isolated dental pulp cells. Yan et al. (2011) modified the enzyme digestion method by utilizing a size-sieved protocol in order to get small-sized cell populations containing a high percent of stem cells (Yan et al., 2011). The

alternatively developed colony culture method is not only time-consuming, but also obtained colonies are not uniform (Gronthos et al., 2000).

The other and commonly used techniques are magnetic and fluorescent-activated cell sorting (FACS). While magnetic-activated cell sorting seems to be simple and inexpensive, FACS offers high purity and viability. However, electrical charges applied to the cell, resultant semi-sterility, and high cost are the drawbacks of the fluorescent-activated cell sorting method (Tirino et al., 2011). Since the STRO-1 antibody identifies a cell surface antigen expressed by the osteogenic fraction of stromal precursors in the human bone marrow, it has been used to select the stromal cell precursor of pericyte cells within dental pulp. While the STRO-1^+ fraction can give cells with both odontogenic and multilineage potential in rat dental pulp (Yang et al., 2007), Shi and Gronthos have shown that STRO-1^+ DPSCs express vascular antigen CD146, α-smooth muscle actins, and a pericyte-associated antigen (3G5), by immunohistochemistry, FACS, and/or immunomagnetic bead selection (Shi & Gronthos, 2003).

Laino et al. (2005) showed that c-kit^+/$CD34^+$/$CD45^-$ DPSCs in vitro were capable of woven bone tissue formation (Laino et al., 2005). Even if CD34 is considered a marker for hematopoietic progenitors, Laino et al. chose to use CD34+ cells instead of STRO-1. This decision was based on the fact that STRO-1 only detects 5% of CD34+ stem cells (Simmons & Torok-Storb, 1991), and CD34 positivity not only indicates primitive pluripotential stem cell precursors but also comprises up to 3% of the total population (Zhan et al., 2004).

Furthermore, Hoechst 3342-sorted SP cells from porcine dental pulp have been shown to possess multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis (Iohara et al., 2006). The same group compared the $CD31^-$ $CD146^-$ and $CD31^+$ $CD146^-$ SP cells and found that the former subfractions showed a stronger neurogenic potential, while the adipogenic, chondrogenic, and odontogenic differentiation potential was similar in the two subfractions of SP cells. Moreover, they have shown the subfraction demonstrating $CD34^+$ and vascular endothelial growth factor-2 (VEGFR2)/$Flk1^+$ promoted vasculogenesis in models of mouse hindlimb ischemia (Iohara et al., 2008).

7.2 Cell Culture Media for DPSCs

A medium can influence the manipulation and culture techniques as well as morphology and differentiation ability of the cells especially for the culture of heterogeneous cell populations that contain cells at various stages of differentiation as in dental pulp. DPSCs are usually cultured in Eagle's basal medium (EBM), Eagle's minimum essential medium (MEM), α-MEM, or Dulbecco's modified Eagle's medium (DMEM). A more complex Ham's F12 nutrient medium (F12) was optimized for cloning. A 1:1 mixture of DMEM and F12 combines the richness of F12 and the higher nutrient concentration of DMEM. These support the expansion of the mesenchymal cells without inducing early senescence and differentiation (Freshney, 2005).

It has been shown that various media favor different cell populations in cultures of pulpal cells. While Medium 199 favors fibroblastic cells, MEM and BME support many connective tissues and epithelial cells (Miller et al., 1976). Nakashima (1991) reported that the DMEM and the DMEM/F-12 mixture led to the best results in adhesion, cell growth, and cell number at confluence when compared with F-12, Roswell Park Memorial Institute medium (RPMI) and Medium 199 (Nakashima, 1991). Initially Nakashima concluded that DMEM may be preferred for culturing pulp cells, because the resulting population ratio and labeling index showed that it supported a population of the prominently fibroblastic pulp cells. However, later her group used very complex media for porcine deciduous pulp cultures. Even though their enzymatic digestion was not described clearly, the media was EBM2, including growth factors such as basic bFGF, insulin-like growth factor 1 (IGF1), epidermal growth factor (EGF), and VEGF-A. In addition, the optimal concentration of porcine serum was also determined to maintain all the sorted cells (Iohara et al., 2008). Although they used DMEM in their consequent studies, the serum ratio was 2% (Iohara et al., 2008) or 10% (Ishizaka et al., 2013), and supplements were not indicated. Since the medium used for stem cell culture can dramatically influence the outcomes, employing such a broad spectrum of media would make it challenging to compare data even within the same research group.

Lopez-Cazaux et al. (2006) demonstrated that calcium-rich, phosphate-poor MEM medium supported the recruitment and/or proliferation of smooth muscle actin-positive (SMA+) cells in human dental pulp cultures, while high-calcium RPMI 1640 medium decreased SMA+ cells. They further reported that MEM was more effective than RPMI 1640 in promoting odontoblast-like cell differentiation (Lopez-Cazaux et al., 2006). Govindasamy et al. (2010) cultured dental pulp cells in four different media: DMEM-knockout (DMEM-KO), DMEM-low glucose (DMEM-LG), DMEM/F12, and minimum essential medium-alpha (α-MEM), all supplemented with 10% FBS. They observed that α-MEM and DMEM-KO are the most optimal culture conditions for DPSC in terms of their proliferation, morphology, cell surface marker analysis, and population doubling time. The interesting result from this study is that while cells grown in α-MEM, DMEM-KO, and DMEM/F12 showed differentiation into mesoderm lineages at early passage (P1) and late passage (P9), DPSC cultured under DMEM-LG has been reported to exhibit differentiation defects when the cells were differentiated after passage 5 (Govindasamy et al., 2010).

In our laboratory we use the outgrowth method with DMEM/F12 plus 10% FBS for both SHED and DPSC: starting from their first days in culture, DPSCs show the spindle-shaped, typical fibroblast-like appearance that is consistent through several passages (over 35) (Fig. 7.2).

Kerkis and Caplan (2012) stressed that the composition of cell populations obtained from in vitro isolation and culture is influenced by the chosen isolation method and the conditions of the cell culture environment. Factors such as the amount and type of fetal bovine serum (FBS) in the culture medium, the use of enzymatic digestion, the inclusion of growth factors, and other modifications to the isolation protocol can all impact the selection or growth of specific populations of

Fig. 7.2 Typical fibroblastic-like appearance of DPSC. (inverted phase-contrast microscopy, ×400)

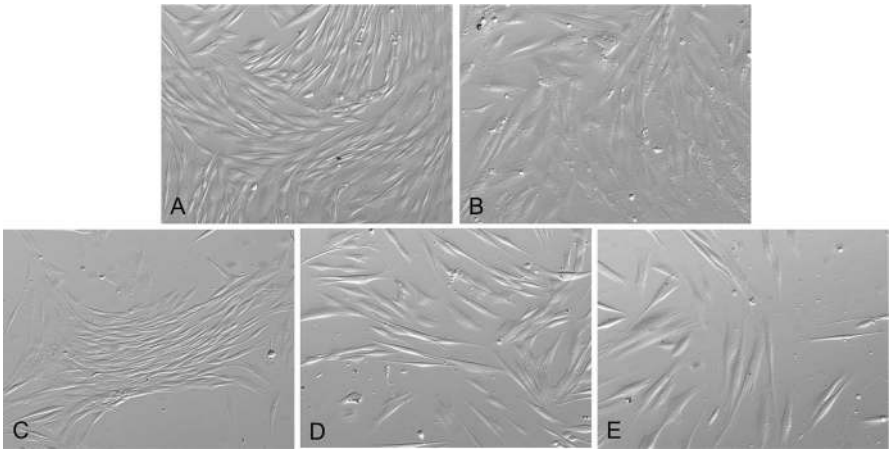

Fig. 7.3 The effects of albumin on cell behavior. (**A**) Serum-free media including 0.05% albumin, (**B**) DMEM/F12, and (**C**–**E**) serum-free media +1%, 0.5%, and 0.1% human serum albumin, respectively (inverted phase-contrast microscopy, ×200, unpublished observation)

stem cells and their progenitors (Kerkis & Caplan, 2012). Likewise, amino acid, protein, glucose, and vitamin contents of media might be responsible for different outcomes. For example, our preliminary data on the effects of albumin on DPSC proliferation indicate that albumin strongly influences cell morphology, inducing bridge-like connections between cells without affecting their elongated fibroblastic appearance or proliferation (Fig. 7.3).

In a nutshell, the techniques used to obtain DPSCs differ widely, even within the same group. These variations make comparison difficult: neither the same techniques nor cell culture media being used consistently (Table 7.2). Although very widely screened data is available within the literature with regard to the cellular and molecular characteristics of expanded cell cultures, different techniques definitely cause different and more confusing results. Therefore, the interpretation of the data needs careful evaluation. Researchers might focus on their needs in published data, not only on already interpreted results.

The choice of supplements for culturing DPSCs in vitro is a crucial factor. Fetal bovine serum (FBS) is the most frequently used supplement, followed by fetal calf serum (FCS), human serum, and a combination of FCS and FBS. A study by Ferrúa et al. (2017) revealed that most research supplemented the culture medium with two to four components besides serum, with penicillin and streptomycin being the most common, accounting for over half of all supplements used (Ferrúa et al., 2017).

7.3 Cryopreservation of DPSC

Cryopreservation is a process where cells or whole tissues are preserved by cooling to very low sub-zero temperatures (typically −196 °C). This allows cells to re-start proliferation, differentiation, and new tissue formation for therapeutic use. At these low temperatures, any biological activity, including the biochemical reactions that would lead to cell death, is effectively stopped. However, when cryoprotectant solutions are not used, the cells being preserved are often damaged during freezing or thawing. Cryoprotectants (CPAs) are substances added to cells before freezing to enhance their survival after thawing. The composition of the CPA solution is a critical factor in optimizing the recovery of cell-based products from cryopreservation. Dimethyl sulfoxide (DMSO) is the most widely used CPA, but its potential toxicity has raised concerns. A review of clinical trials reporting DMSO-related adverse effects suggests that these issues may be attributable to incomplete DMSO removal or procedural errors and could potentially be avoided with proper precautions (Gurruchaga et al., 2018; Lee et al., 2012). It has been demonstrated that exposure to a static magnetic field can enhance the survival rate of DPSC during cryopreservation without the use of DMSO. This protective effect is potentially attributed to increased membrane stability, which helps the cells resist damage caused by ice crystals during the freezing process (Lin et al., 2015). Conventional, multi-step slow freezing and vitrification methods were comparatively tested, and an efficient and reliable cryopreservation method for DPSC resulting in the high cell survival rate has been reported (Chen et al., 2011; Hilkens et al., 2016; Lee et al., 2010; Lindemann et al., 2014; Papaccio et al., 2006; Perry et al., 2008; Woods et al., 2009). However, ice crystal formation is inevitable throughout the sample during slow freezing. Studies on intracellular ice formation in confluent DPSC monolayers have shown that these cells express Connexin-43, a protein involved in gap junction

Table 7.2 Different techniques for DPSC and SHED isolation and growth

Group	Isolation	Growth medium	Selection	Differentiation into	Plus
DPSC (Gronthos et al., 2000)	3 mg/ml collagenase type I/4 mg/ml dispase	α-MEM + 20% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S		Osteogenic cells	Characterization of the immune-phenotype and generation of a dentin-like structure lined with human odontoblast-like cells that are surrounded by a pulp-like interstitial tissue in immunocompromised mice
DPSC (Liu et al., 2006)	3 mg/ml collagenase type I/4 mg/ml dispase	α-MEM + 10% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S	Magnetic STRO-1$^+$, 3G5$^+$, CC9$^+$	Osteogenic, Adipogenic, Neurogenic cells	
DPSC (Honda et al., 2007)	Tissue explants	α-MEM+ 10% FBS, 1× Glutamax, 50 μg/ml L-ascorbic acid phosphate magnesium salt n-hydrate, P/S	FACS Hoechst 33342	Osteogenic cells	Expression of: ABCG2, Nestin, Notch-1, α-SMA
DPSC (Spath et al., 2010)	Trypsin pre-treated tissue explants	MegaCell DMEM complete medium +10% FCS, 2 mM L-glutamine, 0.1 mM β-mercaptoethanol, P/S		Osteogenic, Chondrogenic, Myogenic cells	Contribution of human DPSC to regenerating muscle in mice
DPSC (Ishizaka et al., 2012)	Specific enzyme is not identified	DMEM +10%FBS	FACS CD31$^-$	Angiogenic, neurogenic cells	Regeneration of the pulp tissue in pulpectomized root canals in dogs
SHED (Miura et al., 2003)	3 mg/ml collagenase type I/4 mg/ml dispase	α-MEM + 20% FCS, 100 μM L-ascorbic acid 2-phosphate, 2 mM L-glutamine, P/S		Neural, Adipogenic, Odontogenic cells	After in vivo transplantation, induction of bone formation, generation of dentin, and survival in the mouse brain along with the expression of neural markers
SHED (Kerkis et al., 2006)	3 mg/ml collagenase type I/4 mg/ml dispase	(DMEM)/Ham's F12 (1:1) + 15% FBS, 2 mM L-glutamine, and 2 mM nonessential amino acids, P/S		Neuronal, Chondrogenic, Osteogenic, Myogenic cells	Expressions of embryonic stem cell markers Oct4, Nanog, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81

SHED (Bakopoulou et al., 2011)	3 mg/ml collagenase type I/4 mg/ml dispase	α-MEM + 15%FBS, 100 μM L-ascorbic acid, 2 mM L-glutamine, P/S, 0.25 mg/ml amphotericin B		Odontogenic and osteogenic cells	
SHED (Bakopoulou et al., 2011)	Tissue explant	DMEM+10% FBS, P/S, 0.25 mg/ml amphotericin B		Odontogenic and osteogenic cells	
SHED (J. Wang et al., 2010)	0.3 mg/ml collagenase type I/0.1% dispase II	DMEM+15% FBS, P/S,		Osteogenic, adipogenic cells	Enhanced potential to form bone after transplantation with ceramic bovine bone into subcutaneous of immunocompromised mice

DPSC Dental pulp stem cell, *SHED* Stem cells from human-exfoliated deciduous teeth, *α-MEM* Alpha modification of Eagle's medium, *DMEM*: Dulbecco's modified Eagle's medium, *FBS* Fetal bovine serum, *FCS* Fetal calf serum, P/S: 100 U/ml penicillin, 100 μg/ml streptomycin

formation. While the cells maintain membrane integrity after intracellular ice formation, they lose their ability to proliferate (Zhurova et al., 2010).

Only one review claimed that dental pulp tissues of cryopreserved teeth are not able to maintain their biological properties because of the limitation of permeability of cryopreservative agent into pulp cavity (Osathanon, 2010); however, Pitak-Arnnop et al. highlighted some technical limitations in this review and suggested caution in interpreting its findings due to potential pitfalls and limitations (Pitak-Arnnop et al., 2010). Accordingly, studies have demonstrated that cryopreserved whole teeth are a viable source of DPSCs for autotransplantation (Lee et al., 2010). Notably, even after 11 years, autotransplantation of a cryopreserved tooth yielded satisfactory results in terms of viability and functional (Haneda et al., 2024). My personal experience also suggests that DPSCs can be isolated from teeth after cryopreservation.

Wang et al. (2022) investigated a method for immediate cryopreservation of whole teeth at −80 °C after extraction. Their study compared the survival rates, morphology, proliferation rates, cell activity, surface antigens, and differentiation abilities of DPSC isolated from fresh teeth with those isolated from teeth cryopreserved for 1 month in either 5 or 10% DMSO. The results showed that this new cryopreservation approach did not negatively impact the overall capabilities or stemness of DPSC, except for a slight delay in the appearance and a reduced recovery rate of DPSC from teeth cryopreserved in 10% DMSO (Wang et al., 2022).

A systematic review revealed that the cryopreservation process for DPSCs involves six steps: dental element disinfection, pulp extraction, cell isolation, cell proliferation, cryopreservation, and thawing. The majority of studies demonstrated high cell viability and proliferation rates after thawing for both primary and permanent teeth, particularly when using DMSO, programmable freezing, and storage in liquid nitrogen. Additionally, the reviewed methods did not impact cell differentiation capacity or the characteristic fibroblastic morphology of mesenchymal cells (Paes et al., 2021). Though cryopreservation methods are currently sufficient for cell research purposes, in my opinion more concrete and successful clinical evidence of their indispensable benefits for whole tooth and isolated DPSC banking facilities for future regenerative purposes is required.

Although it is still unclear whether any of the MSC can be used for future cell therapies, establishing stem cell banks containing pluripotent cells which closely match or are compatible to HLA, along with the induction of antigen-specific immunological tolerance instead of requiring lifelong immunosuppressive therapies, are hopes for the avoidance of immunological rejection (Taylor et al., 2011). There are efforts to establish clinical-grade DPSC banks with a sufficient repertoire of HLA types. Tamaoki et al. used DPSC dental pulp stem cells to generate iPSC banking with a sufficient repertoire of HLA types. The practical isolation and handling of dental pulp cells may make it easy to expand the size of the bank in multiple institutes (Tamaoki et al., 2010).

7.4 Differentiation Regimens for DPSC

Recalling the defining characteristics of DPSCs, cells must meet three key criteria, according to the International Society for Cellular Therapy (ISSCR), to be classified as mesenchymal stem cells:

1. They must adhere to plastic surfaces when cultured under standard conditions.
2. They must express specific surface markers like CD105, CD73, and CD90 while lacking others like CD45, CD34, CD14, CD11b, CD79alpha, CD19, and HLA-DR.
3. They must be able to differentiate into osteoblasts, adipocytes, and chondroblasts when grown in a laboratory setting (Dominici et al., 2006).

As detailed in the first chapter of the book, the criteria reported by ISSCR as the in vivo characterization of MSCs do not provide evidence that these cells are bona fide stem cells. For example, after the generation of iPSCs, pluripotency is evaluated according to the similarity of putative cells to ESCs. Along with exploring the expression of ESC-associated antigens and pluripotency genes, a teratoma assay should be performed to confirm pluripotency in vivo (Yildirim, 2012). Similarly, to confirm the presence of true MSCs in the bone marrow, these cells should be transplanted to ectopic sites to determine their ability to form ossicles (Bianco et al., 2013). As mentioned in Chap. 5, DPSCs are not the stem cells of the dental pulp. Instead, they are mesenchymal stromal cells derived from the dental pulp.

7.4.1 Recipes for Tri-lineage Differentiation of DPSC

The classical differentiation recipes are as follows: Osteogenesis can be initiated experimentally with DMEM-LG (low glucose) with 10% (vol/vol) FBS, 100 nM dexamethasone, 0.2 nM ascorbic acid, and 10 mM β-glycerophosphate; in the case of chondrogenesis, 10 ng/ml TGFβ3, 100 nM dexamethasone, 50 μg/ml ascorbic acid, 1 mM sodium pyruvate, 6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml bovine serum albumin, and 5.35 mg/ml linoleic acid in DMEM-high glucose can be used. Last but not least, adipogenesis can be initiated with the xenobiotics such as 1 μM dexamethasone, 500 μM IBMX, 60 μM indomethacin, and 5 μg/mL insulin in DMEM-LG supplemented with 10% FBS. Oil red O staining of 4% PFA-fixed cells and toluidine blue staining in cryosections of cell nodules in micropellet systems are universally accepted visualization methods for lipid droplets in adipogenically induced cells and accumulation of extracellular matrix composed of mucopolysaccharides in chondrogenically induced cells, respectively (Fig. 7.4) (Akhan et al., 2015; Oktar et al., 2011; Yildirim et al., 2012).

Despite some pitfalls in von Kossa and Alizarin Red S (Bonewald et al., 2003; Wang et al., 2006), they are still in use for expected collagenous extracellular matrix formation and mineralization in osteogenically induced cells (Deegan et al., 2014).

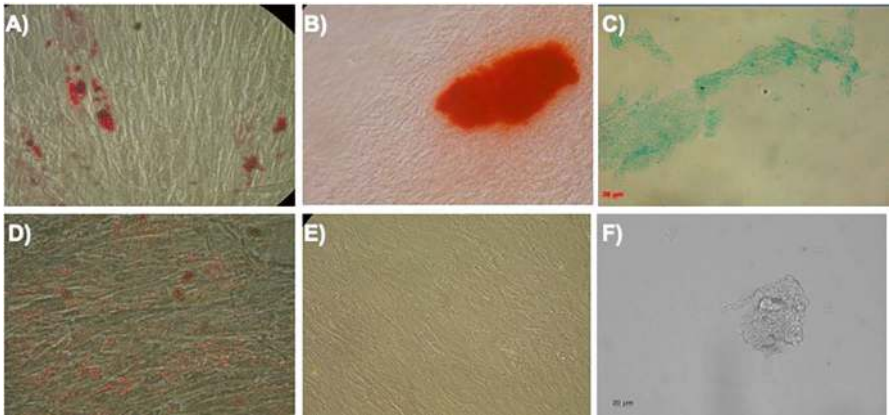

Fig. 7.4 Adipogenic (**A**), osteogenic (**B**), and chondrogenic (**C**) differentiation of dental pulp stem cells. Cells cultured in control media (**D**–**F**). Oil red O staining for adipogenic differentiation, Alizarin Red staining for osteogenic differentiation, and alcian blue staining for chondrogenic differentiation (Akhan et al., 2015)

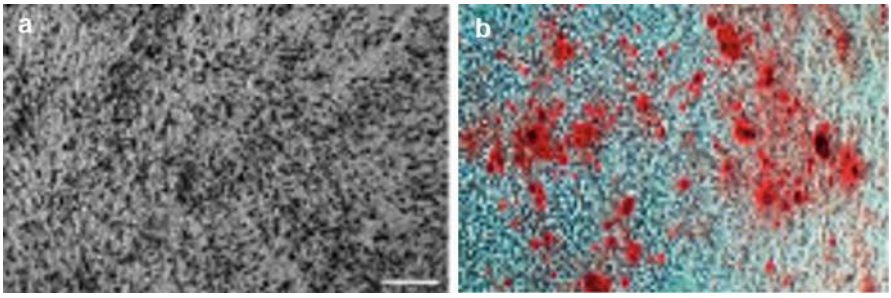

Fig. 7.5 Results of the osteogenic differentiation induction of DPSC. While von Kossa shows only calcium accumulations (**a**), Alizarin Red S reacts with calcium cations to form a chelate (**b**) (Yildirim et al., 2012)

While von Kossa shows only calcium accumulations (Fig. 7.5a), Alizarin Red S reacts with calcium cations to form a chelate (Fig. 7.5b). Although other stains such as two fluorescent dye combinations (xylenol orange and alcian blue) have been suggested, staining methods alone are not appropriate for the identification and quantification of bonelike minerals. Hence, using more specific diagnostic techniques, such as X-ray diffraction, electron microscopy, or Fourier transform infrared spectroscopy (FTIR), may better verify the presence and quality of calcium phosphate phases (Bonewald et al., 2003).

On the other hand, the pattern of von Kossa staining in osteogenically differentiated DPSCs differed significantly. We observed that DPSCs exhibited a more diverse distribution of small, round, darkly stained mineralized structures (Fig. 7.6a), while human umbilical cord stem cells (hUCSCs) displayed bone nodule-like formations (Fig. 7.6b).

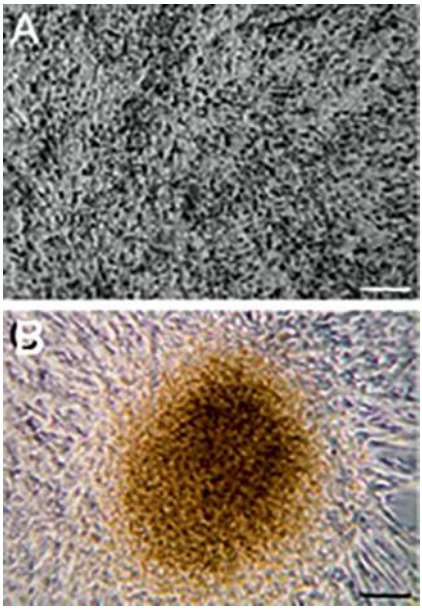

Fig. 7.6 Difference in von Kossa staining in osteogenically differentiated DPSC (**a**) and human umbilical cord stem cells (**b**). Scale bars: 100 μm

7.4.2 Problems in Adipogenesis

While most of the differentiation assessments revealed a strong osteogenic and chondrogenic potential for DPSC (Gronthos et al., 2000, 2002; Honda et al., 2007; Huang et al., 2006; Iohara et al., 2006; Kerkis et al., 2006; Liu et al., 2006), there are no consistent reports of their adipogenic potential (Gronthos et al., 2000; Oktar et al., 2011; Pivoriūnas et al., 2010; Struys et al., 2011). We compared the differentiation potentials of human DPSC and human umbilical cord stem cells (hUCSCs). While chondrogenic and osteogenic differentiation of both cell types were similar, their adipogenic differentiation within the same conditioned media resulted in different outcomes. While hUCSC started to display specific intracytoplasmic lipid granules in the central cytoplasm after only 48–72 h following adipogenic treatment, DPSC protected their fusiform shape without showing any lipid granules even after prolonged treatments of up to 5 weeks (Fig. 7.7). Furthermore, following successful adipogenic induction of hUCSC, we found that nucleostemin (NS), which has been shown as a nucleolar protein expressed in stem and cancer cells, could no longer be detected in forming preadipocytes. However, DPSC that did not respond to adipogenic induction displayed a stable NS expression (Oktar et al., 2011). Other groups also noted the non-responsiveness of DPSC to adipogenic stimuli. Pivoriūnas et al. (2010) reported the same results for adipogenic differentiation of DPSC after several trials (Pivoriūnas et al., 2010).

Although DPSCs have been reported to possess adipogenic differentiation capacity (Huang, 2009; Koyama et al., 2009), this capacity was not observed under our experimental conditions. This discrepancy may be due to several factors, including

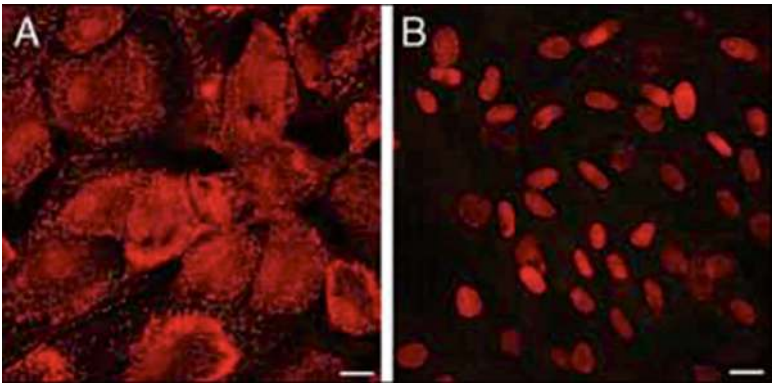

Fig. 7.7 (**A**) Adipogenically induced hUCSCs produced lipid droplets (oil red O-stained cytoplasmic granules emit red fluorescence signals) in association with the transformation of cell morphology from flat to large round cells. hrDPSCs showed no induction (**B**) (nuclei of the cell, red signal). Scale bars: 20 μm (Oktar et al., 2011)

individual differences. The environmental conditioning or predisposition of the stem cells might be reflected in their distinctive differentiation ability. Their fate in vivo might be dependent of their embryonic origin and their anatomical localization. Moreover, teeth are derived from the ectoderm of the first branchial arch and the ectomesenchyme of the neural crest. Deciduous teeth start to form between the sixth and eighth weeks of gestation, and permanent teeth begin to form in the twentieth week (Thesleff et al., 1995). Umbilical cord, on the other hand, develops from the extra-embryonic mesoderm at day 12–13 after fertilization. Likewise, Struys et al. (2011) claimed that the dental pulp is confined by a hard dental tissue (dentin) which is more likely to create a tendency for osteogenic events in those cells (Struys et al., 2011). However, the observed strong osteogenic response of umbilical cord intervascular cells in our experimental conditions does not support this hypothesis (Yildirim et al., 2012). Therefore, the diverse developmental lineage and differential potential of UCSC and DPSC may reflect stem cell heterogeneity or epigenetic discrepancy. It has been suggested that the propensities of MSCs derived from various tissue sources toward a specific lineage are different from each other (Kerkis & Caplan, 2012). Regarding the possibility that the epigenetic regulation probably depends on the stem cell origin, we have also investigated genetic and epigenetic mechanisms to explain the variations of the differentiation of DPSCs into adipocytes. We examined the expression of stem cell and epithelial-mesenchymal transition (EMT) markers as well as histone modification enzymes (H3K4me, H3K4me3, H3K9me2, H3K9me3, H3K27me2, H3K27me3, H3K36me2, H3K36me3) as well as methyltransferases and demethylases such as set 7/9, G9a, EzH2 and JARID1A, JMJD1, and JMJD both with western blot and immunofluorescence. We found a significant difference in the expression of H3K4me, H3K36me3, and set 7/9, JMJD1, and JMJD2 in DPSCs that have different an adipogenic differentiation potential. Hence, the differentiation of DPSCs can be modulated by the factors that

affect epigenetic alterations and can therefore be useful in directing the differentiation of DPSCs (Akhan et al., 2015). Likewise, Argaez-Sosa et al.'s research suggests that elevated expression of DNMT1, DNMT3A, TET1, and TET2 genes may hinder the differentiation of DPSCs into adipocytes, but not PLSCs. This observation aligns with the downregulation of adipogenesis-related genes (KLF4, c-MYC, and PPARγ) in DPSCs (Argaez-Sosa et al., 2022).

Essentially, in most cases, when analyzing individual cells within a MSC population, only a small proportion demonstrates the ability to differentiate into multiple cell types (biopotential) or even a single-cell type (unipotential) (DiGirolamo et al., 1999; Javazon et al., 2004).

7.5 Odontogenic Differentiation

Spontaneous differentiation of DPSC toward several lineages has been shown (Kerkis & Caplan, 2012; Lizier et al., 2012; Paino et al., 2010; Yu et al., 2010). Given the heterogeneous nature of DPSC cultures, it is possible that odontoblasts may not be among the lineages that emerge under standard growth and proliferation conditions, as these conditions may not provide the necessary signals for odontoblast terminal differentiation. Papaccio's group has shown that in the presence of 20% of FBS, the bone is the main commitment of DPSCs, but not dentin (d'Aquino et al., 2007; Laino et al., 2005, 2006; Papaccio et al., 2006). On the other hand, DPSC and SHED have been shown to have an odontogenic potential with several different in vitro and in vivo evaluation methods, in addition to quite different isolation, culturing, and differentiation methods (Table 7.3). Furthermore, there are currently no specific markers to definitively distinguish between odontoblasts and osteoblasts. While beta-glycerophosphate, ascorbic acid, and dexamethasone have been commonly used to induce odontoblast differentiation, researchers continue to explore novel methods for achieving this process in vitro (Kawata et al., 2021; Nakazato et al., 2024; Narwidina et al., 2023; Park et al., 2020; Wang et al., 2023; Wu et al., 2019; Xiao et al., 2021; Zheng et al., 2020, 2023).

Many other factors have been shown to promote odontoblastic differentiation of DPSC, such as porcine tooth germ conditioning medium (Wang, Ma, et al., 2011b); the combination of MTA and enamel matrix derivatives (Min et al., 2009); FG2, TGFβ1, BMP-2, GDF-11, DMP-1-derived peptide, asporin, and EphB/ephrin-B (Casagrande et al., 2010; Chaussain et al., 2009; He et al., 2008; Iohara et al., 2004; Nakashima et al., 2004; Yang et al., 2007, 2009); inhibition of delta 1 (Wang, He, et al., 2011a); TWIST1 overexpression (Li et al., 2011); Bmi-1 transduction (Mehrazarin et al., 2011); the inhibition of mammalian target of rapamycin (mTor) complex Torc 2 (Kim et al., 2011); pro-inflammatory cytokines(IL-1 and TNF) (B. Yang et al., 2012); simvastatin (Okamoto et al., 2009); conditioned medium extracted from tooth germ cells (Duailibi et al., 2004; Ohazama et al., 2004); natural mineralized scaffolds (Wang et al., 2006; Zhang et al., 2012); and even oral bacterial extracts (Abe et al., 2010).

Table 7.3 Evaluation of odontogenic differentiation by different research groups

Group	Odontogenic induction by	Evaluation markers	Evaluation methods	Results
Couble et al. (2000)	Culturing DSPC in EBM supplemented with 10% or 15% FCS ± β-GP	Coll type I, DSP	Electron microscopy, X-ray microanalysis, RT-PCR	Inducing odontoblastic features including spatial organization similar to odontoblastic layer
Gronthos et al. (2002)	Transplantation of DPSC with HA/TCP ceramic powder subcutaneously into immunocompromised mice		Histology	Generation of ectopic dentin/pulp-like complexes
Batouli et al. (2003)	Transplantation of DPSC with HA/TCP ceramic powder subcutaneously into immunocompromised mice	DSP	Histology, histochemistry	Formation-vascularized pulp-like tissue surrounded by a layer of odontoblast-like cells expressing DSP
G. Huang et al. (2006)	DPSC cultured in α-DMEME including %20 FBS 2 mM KH2PO4, 20 mM HEPES, and 100 nM dexamethasone; or these three chemicals plus 5×10^{-8} M Vit D3		Alizarin Red S staining	Promoted mineral nodule formation
Cordeiro et al. (2008)	SHEDs seeded in dentin slice/PLLA scaffolds were transplanted into immunodeficient mice	Human factor VIII, DSP,	Histochemistry, B-galactosidase staining, TEM	Pulp-like tissue with functional blood vessels lined with odontoblastic cells
Nam et al. (2011)	DPSCs were populated on porous calcium phosphate granules	DSPP, DSP, Col type I, DMP1, OCN	RT-PCR, WB, Alizarin Red S staining	Odontogenic development supported by the upregulation of DSPP, DMP-I, Coll type I, and OCN
Lee et al. (2011a)	Pre-ameloblast-derived factors	DSPP, BSP, nestin, Coll type I, RUNX2, ALP, OC, mCpne7	Real time PCR, WB, Alizarin Red S staining, dentin sialophosphoprotein promoter activity, liquid chromatography-mass spectrometry in vivo: transplantation into immunocompromised mice	Facilitated odontoblast differentiation and generation of pulp-like structures lined with human odontoblast-like cells showing typical odontoblast processes

BME Eagle's basal medium, *Coll type I* collagen type I, *OCN* osteocalcin, *DSP* dentin sialoprotein, *DSPP* dentin sialophosphoprotein, *DMP-1* dentin matrix protein-1, *β-GP* beta-glycerophosphate

Since mechanical forces have effects on the differentiation of MSC (Bayati et al., 2011; Sarraf et al., 2011), there are also reports that evaluate behavioral differences under mechanical stress. While Yu et al. (2009) showed odontoblastic differentiation potential of DPSC is enhanced under dynamic hydrostatic pressure (Yu et al., 2009), Cai et al. (2011) reported that uniaxial cyclic tensile stretch inhibits osteogenic and odontogenic differentiation of DPSC (Cai et al., 2011). The effect of oxygen tension on proliferation and differentiation of DPSC has been surrogated and shown that hypoxic conditions (3% O_2, 5% CO_2, and 92% N_2) increased the proliferation but inhibited the differentiation capacity to odontoblasts (Iida et al., 2010). For a detailed review of the effects of different signaling pathways on odontogenic differentiation of dental pulp stem cells, please refer to Zhou et al. (2023) (Zhou et al., 2023).

Although the mechanism of odontoblastic differentiation is not known, many researchers are doing further investigations in order to discover any odontoblast-specific gene expressions of those morphologically odontoblast-like cells. As of August 2024, a PubMed search for "odontoblast differentiation" yields 2284 studies. In today's world of increasingly complex molecular biology techniques, it is not only challenging to stay current with all research but also to translate findings into practical applications. The absence of multidisciplinary teams further hinders the transition of these remarkable discoveries into clinical settings. My hope lies in the potential of young researchers to harness artificial intelligence and apply this data to develop effective treatments.

7.5.1 Is There a Predetermined Cell Fate to Become Either an Osteoblast or an Odontoblast?

Examining tooth development in Runx2 transgenic mice, Miyazaki et al. showed that Runx2 not only inhibits the terminal differentiation of odontoblasts but also induces transdifferentiation of odontoblasts into osteoblast (Miyazaki et al., 2008). Runx2, also known as core-binding factor subunit alpha-1, is a key transcription factor associated with osteoblast differentiation (Komori, 2010, 2011). Although it is essential in tooth development and abundantly expressed in the dental pulp (S. Chen et al., 2009; Yildirim et al., 2008) and PDL tissues (Bağ & Yildirim, 2017), its function in odontoblast differentiation is unclear. The role of Runx2 in the regulation of odontoblast differentiation is hypothesized by Komori (Komori, 2010, 2011) (Fig. 7.8).

Moreover, it has been shown that TWIST-1 and TWIST-2 suppress the activity of Runx2 and thereby regulate bone formation (Bialek et al., 2004; Kronenberg, 2004). Likewise, Li et al. showed that overexpressing or silencing TWIST1 levels could tune the differentiation capacity of DPSC effectively (Li et al., 2011). In this sense, TWIST1 and RUNX2 are seen to be unique candidates for probing odontoblast

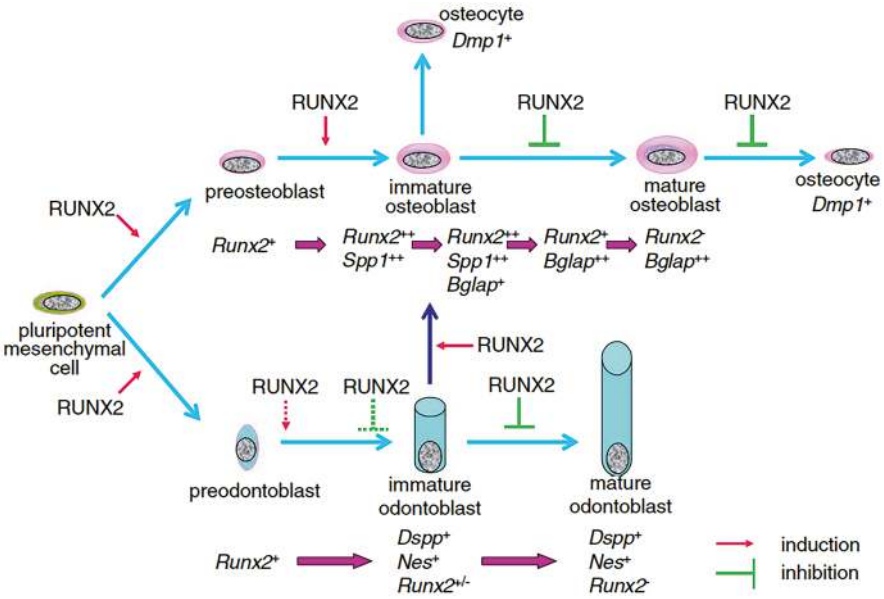

Fig. 7.8 Regulation of osteoblast and odontoblast differentiation by RUNX2. RUNX2 directs pluripotent mesenchymal cells to the osteoblast lineage and increases the number of immature osteoblasts but inhibits osteoblast maturation. Preosteoblasts express Runx2. Immature osteoblasts express Runx2 and Spp1 and, subsequently, Bglap. Mature osteoblasts express Bglap, but Runx2 expression is downregulated. Osteocytes express Dmp1. The transition of immature osteoblasts to osteocytes occurs at an early stage of bone development. The common precursors of osteoblasts and odontoblasts are restricted to neural crest-derived mesenchymal cells, but the basal process of osteoblast differentiation is similar in the neural crest-derived and non-neural crest-derived pluripotent mesenchymal cells. Preodontoblasts differentiate from neural crest-derived pluripotent mesenchymal cells. RUNX2 is essential for differentiation of pluripotent mesenchymal cells into preodontoblasts. RUNX2 also probably induces the differentiation of preodontoblasts into immature odontoblasts at an early stage but is inhibitory at a late stage. Preodontoblasts express Runx2, immature odontoblasts express Dspp and Nes but Runx2 weakly, and mature odontoblasts express Dspp and Nes but not Runx2. Runx2 expression is downregulated during odontoblast differentiation, and RUNX2 inhibits terminal differentiation of odontoblasts. Overexpression of Runx2 induced transdifferentiation of odontoblasts to osteoblasts (Spp1-secreted phophoprotein 1/osteopontin, Bglap bone gamma carboxyglutamate [gla] protein/osteocalcin, Dspp dentin sialophosphoprotein, Nes nestin, Dmp1 dentin matrix protein 1). (Reproduced from (Komori, 2010) with the permission of the publisher)

lineage commitment, as Sui Huang group's pioneering studies confirmed that lineage specification of multipotent progenitor cells is governed by a balance of lineage-affiliated transcription factors (S. Huang et al., 2007; Zhou et al., 2011). In developmental or repair events, the progenitor cell(s) of the dental pulp can be directed in the choice between osteoblast and odontoblast fates via RUNX2 or TWIST-1 couples.

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Chapter 8
Reprogramming of DPSC to Induced Pluripotent Stem Cells

A major goal in cell biology research is achieving a genuine regenerative response from pluripotent cells, which can differentiate into nearly any cell type. In a multicellular organism (metazoan), all cells typically possess the same set of genes. Exceptions to this rule include post-meiotic germ cells, mature lymphocytes, and cells in species that undergo chromosome diminution (Kloc & Zagrodzinska, 2001). Therefore, generating pluripotent cells in vitro and guiding their differentiation into specific cell types—essentially "rewinding" any mammalian cell to an embryonic state and then "forwarding" these versatile cells toward diseased tissues—represent a logical and promising approach in regenerative medicine. In 2006, Takahashi and Yamanaka published a landmark strategy using only four transcription factors (OCT4, SOX2, KLF4, and c-Myc) to induce reprogramming of somatic mammalian cells into a pluripotent state, known as induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006). Since iPSCs avoid the ethical and legal issues associated with embryonic stem cells (ESCs), this technology has a profound impact on clinical applications. These cells have not only challenged long-held beliefs about terminally differentiated cells in developmental biology but also illuminated how cells harness their inherent potential to generate other cell types. The last few years have seen a continuous effort to derive therapeutically safe iPSCs.

By pushing the boundaries of conventional biology, iPSCs have ushered in a new era for biologists, who now require more than words but tangible formulas derived from the fundamental laws of mathematics and physics to understand the complex and emergent behavior of lineage commitment and pluripotency (Huang, 2009). Research on regaining pluripotency and directing cells into the desired differentiated states has yielded data that now offers exciting alternatives in areas such as disease pathogenesis, drug discovery, oncology, and other potential applications.

S. Yildirim, *Dental Pulp Derived Mesenchymal Stromal Cells*,
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8.1 A Brief History of Efforts to Change Somatic Fate

Although cell fates during development are neither fixed nor irreversible, the deeply rooted belief in cell biology was that terminally differentiated cells lose the potential to produce other cell types (Huang, 2009). The first study demonstrating otherwise showed that nuclei of frog blastula cells could develop into complete embryos when transplanted into enucleated embryos (Briggs & King, 1952). A few years later, Gurdon et al. (1958) reprogrammed fully differentiated intestinal cells from *Xenopus* using frog oocytes (Gurdon et al., 1958). It took another two decades before Evans and Kaufman successfully isolated embryonic stem cells (ESCs) in 1981 (Evans & Kaufman, 1981). While changing somatic cell fate was achieved using frog oocytes, somatic cell nuclear transfer (NT) remained unsuccessful in other species until the late 1990s. Then, Wilmut et al. (1997) cloned Dolly the sheep (Wilmut et al., 1997). The first successful derivation of human ESCs was reported a year later (Thomson et al., 1998). However, ethical complications, scarcity of human egg cells for research, low efficiency, technical difficulties, and aberrant ploidy made ESC research more controversial than expected (Walia et al., 2012). Accordingly, earlier studies indicated that the "terminally differentiated" state of human cells was not fixed but could be altered, inducing the expression of previously silent genes typical of other differentiated states (Bhutani et al., 2010; Blau, 1992; Blau & Baltimore, 1991; Yamanaka & Blau, 2010). Consequently, pluripotent stem cell lines can be generated directly from adult somatic cells using alternative approaches like nuclear transfer, cell fusion, and direct reprogramming.

Extensive research focused on identifying key transcription factors, hypothesizing that a small set could control cell fate in various contexts, such as reprogramming and transdifferentiation. When a nucleus from a differentiated somatic cell is transplanted into an enucleated oocyte, nuclear reprogramming is triggered, leading to cloning of the original somatic cell. These experiments demonstrated that cell specialization requires only changes in gene expression, not gene content, and this differentiation process can be fully reversed (Yamanaka & Blau, 2010). In 2006, a breakthrough in understanding the main regulators of the ESC state occurred when Shinya Yamanaka and colleagues demonstrated that a combination of just four transcription factors could generate ESC-like pluripotent cells from mouse fibroblasts (Takahashi & Yamanaka, 2006). These generated cells were called induced pluripotent stem cells (iPSCs). The discovery of factor-directed reprogramming had a significant impact on stem cell biology and its potential applications. Today, many fundamental biological concepts are shifting to accept that mature body cells can be reverted to an embryonic state without the need for eggs or embryos (Wilmut et al., 2011).

8.2 Generation of the First iPSC

Takahashi and Yamanaka (2006) demonstrated that overexpressing specific transcription factors could convert cells (or nuclei) into a pluripotent state (Takahashi & Yamanaka, 2006). Their hypothesis was that factors maintaining pluripotency in ESCs might induce pluripotency in somatic cells. They tested 24 transcription factors known for their important roles in mouse ESCs, introducing combinations of these genes into mouse fibroblasts via retroviral transduction. The cells carried an antibiotic resistance gene that would be expressed only when the F-box protein 15 gene (Fboxo15) was activated. This design allowed the antibiotic resistance gene to also be activated if pluripotency was induced by any combination of the 24 candidate genes. Surprisingly, they discovered that only four factors were sufficient to generate ESC-like colonies: octamer-binding transcription factor 4 (Oct4, also known as Pou5f1), SRY-box transcription factor 2 (Sox2), Kruppel-like factor 4 (Klf4), and c-Myc (later collectively termed OKSM). These reprogrammed cells were named induced pluripotent stem cells (iPSCs) (Takahashi & Yamanaka, 2006).

Although the cells selected for their ability to express Fbxo15 shared similarities with ESCs in morphology, growth properties, expression of key ESC marker genes like SSEA-1 and Nanog, and the ability to form teratomas in immunodeficient mice, they also differed in terms of global gene expression profiles and certain DNA methylation patterns. Moreover, they failed to produce adult chimeric mice when injected into early mouse embryos. These characteristics indicated that these "first-generation" iPSCs were not fully reprogrammed. Soon after, using improved endpoints for the reprogramming process, such as selecting for Nanog or Oct4 expression instead of Fbxo15, generated colonies with reactivated pluripotency genes. These cells were more similar to ESCs and could contribute to adult chimeras (Okita et al., 2007). Within a year, human fibroblasts were also reprogrammed using the same transcription factors (Takahashi et al., 2007; Wernig et al., 2007; Yu et al., 2007). Subsequently, several groups independently replicated the reprogramming of human fetal, neonatal, and adult somatic cells into iPSCs (Aasen et al., 2008; Lowry et al., 2008; Park et al., 2008a, b). As an indication that transcriptional pathways of pluripotency are conserved throughout evolution, iPSCs have been generated from various species, including humans, rhesus monkeys, and mice (Li et al., 2009a; Liu et al., 2008). Later, it was demonstrated that iPSC lines could generate mice entirely derived from iPSCs ("all-iPSC" mice) when injected into tetraploid blastocysts (Boland et al., 2009; Gao et al., 2024a, b; Kang et al., 2009; Zhao et al., 2009, 2010).

8.3 Applications of iPSCs

Cellular models derived from iPSCs have become valuable tools for studying the mechanisms underlying various human diseases, including genetic, sporadic, and age-related disorders. Numerous protocols have been established to differentiate iPSCs into various cell types. This is commonly done either by replicating developmental signaling pathways in the laboratory using specific proteins and small-molecule compounds or by overexpressing transcription factors known to control cell fate in order to guide the desired gene expression patterns. Neurons, cardiomyocytes, oligodendrocytes, or T cells can be examples of those protocols (Cao et al., 2014; Iriguchi et al., 2021; Li et al., 2018b; Lin & Zou, 2020; McDonald et al., 2024; Wu & Tang, 2023).

Furthermore, iPSCs and their derivatives are utilized as in vitro models to study the fundamental principles and mechanisms governing cell fate transitions, self-organization, and developmental disorders in humans. These models allow for the testing of potential therapies in a controlled laboratory setting. Using iPSC-derived cells to model human development can offer valuable insights into developmental processes unique to humans, which can then be applied to improve cell differentiation methods for various purposes (Cerneckis et al., 2024; Zernicka-Goetz, 2023).

8.3.1 Disease-Specific iPSCs

The initial focus of therapeutic approaches using iPSCs was on the fact that patient-specific iPSCs offer valuable insights into inherited human disorders. This is because pluripotent stem cells can differentiate into most, if not all, cell types. This concept was heavily based on studies of directed differentiation of subtypes and genetically defined ESCs from animal models (Gearhart, 1998). Furthermore, research on human ESCs has aimed to generate mutant human ESC lines as disease models since Thomson et al. derived human ESC lines in 1998 (Thomson et al., 1998). With known disease-associated genetic loci and specific disease phenotypes, genetically modified human ESCs could contribute to cell replacement therapies and modeling human diseases (Saha & Jaenisch, 2009). However, the use of ESCs has several limitations, including political, religious, ethical, and moral concerns regarding the destruction of human embryos, as well as inefficient methods for generating genetically modified human ESCs. For instance, generating human ESCs via preimplantation genetic diagnosis (PGD) embryos is limited to a few diseases and faces technical challenges. Additionally, only a few monogenic diseases are detectable via PGD, and the severity and clinical symptoms of these diseases vary between patients due to variable penetrance (Colman & Dreesen, 2009). Other alternatives, such as generating individual pluripotent stem cells by nuclear transfer, cell fusion with ESCs, or treatment with extracts of pluripotent cells, are also restricted for various reasons, and only a few diseases have been modeled using

these approaches (Taranger et al., 2005; Wakayama et al., 2001). Animal models have been used to study human diseases for decades, but they also have limitations. These include showing no or only partial resemblance to human disease, differences in physiology and anatomy between animals and humans, inability to mirror cognitive or behavioral defects in neurological diseases, and differences in genetic background between animals and humans that can affect the resulting phenotype of disease-associated mutations (Colman & Dreesen, 2009; Saha & Jaenisch, 2009).

To overcome these drawbacks, iPSCs offer disease- and patient-specific cells with knowledge of the donor's clinical history, and they can be generated from cells taken from individuals of all ages, even elderly patients with chronic diseases (Dimos et al., 2008). Given that human ESC lines show varying abilities to differentiate into specific lineages (Osafune et al., 2008), generating multiple iPSC lines from a single patient is highly advantageous due to their identical genetic background.

Ultimately, disease-specific iPSCs have been generated from individuals with various disorders, including neurodegenerative diseases such as ALS (Dimos et al., 2008; Workman et al., 2023; Yang et al., 2023), Parkinson's disease (Li et al., 2015; Park et al., 2008a; Soldner et al., 2009; Chen et al., 2011, 2021; Xiao et al., 2016), SMA (Ebert et al., 2009; Zeng et al., 2023; Zhou et al., 2018), and Alzheimer's disease (Kondo et al., 2022) and inherited diseases including familial dysautonomia (Lee et al., 2009), adenosine de-aminase deficiency-related severe combined immune deficiency (ADA-SCID), Shwachman-Bodian-Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker muscular dystrophy (BMD), Huntington's disease, juvenile-onset, type 1 diabetes mellitus (JDM), Down syndrome (DS)/trisomy 21, the carrier state of Lesch-Nyhan syndrome (Park et al., 2008a), and Fanconi anemia (Raya et al., 2009; Yung et al., 2013).

A significant limitation of using iPSC-derived cells for modeling neurodegenerative diseases is their lack of age-related characteristics. However, despite this challenge, numerous iPSC-based models have been developed, and new methods to examine age-related events or induce aging-associated phenotypes are being explored (Cerneckis et al., 2023; Chen et al., 2021; Cornacchia & Studer, 2017). Furthermore, a recent progress in modeling neurodevelopmental and psychiatric disorders using iPSC-derived cells is noteworthy due to its potential to lead to more effective treatments (Li et al., 2018a; Wang et al., 2024).

8.3.2 Other Applications of iPSC

The iPSC technology provides a unique tool to investigate how different somatic mutations and other factors can alter the molecular and cellular processes of normal cells, ultimately leading to their transformation into cancer cells (Chehelgerdi et al., 2023; Smith & Tabar, 2019; Zhang et al., 2020a). iPSC-based cell therapy is a promising new approach for repairing or replacing damaged tissues and engineering immune responses to diseases like cancer (Cerneckis et al., 2024). The success of

chimeric antigen receptor (CAR) T cell therapy in treating certain blood cancers has opened the door for developing new cell therapies, including those utilizing iPSC technology (Bashor et al., 2022). Besides, xenotransplantation experiments suggest that iPSC technology can be used to create difficult-to-obtain cell types and restore normal tissue function after transplantation (Balboa et al., 2022; Feng et al., 2023; Jeyagaran et al., 2024; Memon & Abdelalim, 2020; Pazzin et al., 2024). This has led to the initiation of various clinical trials using iPSC-derived cellular products to treat human diseases. These endeavors target a range of diseases, including various cancers where autologous and allogeneic iPSC-based immune cell therapies are in development, genetic developmental disorders requiring cell transplants for tissue repair, and even age-related conditions where tissue replacement is needed. For the most recent detailed information, please refer to Cerneckis et al. (Cerneckis et al., 2024).

Using iPSC-derived cellular models to simulate viral infections, such as COVID-19, can uncover the specific ways viruses interact with human cells (Deguchi et al., 2021; Harschnitz & Studer, 2021; Luo et al., 2021). Additionally, human-derived iPSCs provide a preclinical platform for testing drug efficacy and safety while also revealing human-specific molecular mechanisms of drug action. These advanced human tissue models can further evaluate drug toxicity and pharmacokinetics, serving as a valuable tool for drug screening before human testing. Different somatic cell types, even those difficult to obtain directly, can be produced from disease-specific iPSCs carrying relevant disease-causing mutations or genetic risk factors. This allows researchers to evaluate drug efficacy within the context of a specific genetic makeup (Barak et al., 2022; Cerneckis et al., 2024; Gu et al., 2021; Park et al., 2021).

While autologous iPSC-derived cell therapies offer the potential for gene correction therapies in preclinical animal models (Agrawal et al., 2021; Feng et al., 2024; Lyu et al., 2018; Maxwell et al., 2020; Tang et al., 2022), allogeneic iPSC-derived cell therapies (iPSCs derived from a universal donor) pose a risk of immune rejection. The heterogeneity of iPSCs can influence both their differentiation potential and the immunogenicity of iPSC-derived cellular products. While allogeneic cell therapy offers the potential to simplify the production process, safety concerns, particularly those related to immune rejection, still need to be fully resolved.

In regenerative medicine, iPSCs are being employed to repair or regrow damaged or diseased tissues. This is achieved by creating organ tissues from iPSCs in the lab and transplanting them to the affected area. This therapy shows potential for treating conditions such as blood disorders, musculoskeletal injuries, spinal cord injuries, and liver damage (Gunaseeli et al., 2010; Liu et al., 2011a; Moad et al., 2013; Nori et al., 2011; Suzuki et al., 2013; Yazawa et al., 2011).

8.4 Challenges to Therapeutic Potential of Human iPSCs

iPSC-based therapies are gaining significant traction in modern medicine, with their use in disease modeling, drug screening, and regenerative medicine growing rapidly. On the other hand, quality control and safety are major concerns, and there are several technical challenges in using human iPSCs to treat various irreparable human diseases. Notwithstanding the many advantages of iPSCs, including easy acquisition, the ability to replicate a patient's specific genetic makeup, unlimited cell supply, and reduced immune rejection risks when using a patient's own cells, some factors can hinder their effectiveness in translational tissue engineering. Primarily, due to the use of oncogenes for reprogramming and the inherent genetic instability of this process, there are inherent risks associated with the use of iPSCs in regenerative medicine, including the potential for tumor formation (Yildirim, 2012b). To minimize or eliminate genetic alterations in derived iPSC lines, factor-free reprogramming methods are necessary. Defining a disease-relevant phenotype requires both in vitro and in vivo models. Additionally, gene-targeting strategies are needed to generate markers for differentiation and gene corrections. Furthermore, the development of cell-type-specific lineage reporters, lineage-tracking tools, and tools to disrupt, repair, or overexpress genes is crucial for modeling many human diseases (Saha and Jaenisch 2009). Overall, challenges facing reprogramming technology may include:

- Limitations of traditional reprogramming techniques
- The use of viral vectors, which can pose safety risks
- The need for reliable and reproducible protocols for complete differentiation into specific tissue types
- Variability in the differentiation potential of iPSCs and the limited lifespan of differentiated cells
- The necessity for comparison with ESCs and the lack of appropriate controls
- The potential for aberrations or abnormalities in prolonged cultures
- The difficulty of modeling non-cell-autonomous phenotypes and diseases
- Incomplete maturation of iPSC-derived cells
- Logistics, reproducibility, and the overall cost (Cerneckis et al., 2024; Deinsberger et al., 2020; Zhu et al., 2011)

The recent advancements in using solely chemicals to create iPSCs offer a promising path toward developing standardized, scalable, and efficient methods for reprogramming somatic cells (Guan et al., 2022; Hartley et al., 2024). Given that cellular functions are influenced by microenvironmental stimuli, it is crucial to evaluate the results of iPSC studies in the context of the specific reprogramming method, culture conditions, and differentiation protocols used, as all of these factors can significantly impact the outcome (Daley et al., 2009; Scesa et al., 2021).

The potential lack of immunogenicity in custom-made, patient-specific adult cells derived from iPSCs held a great promise for their use in treating a wide range of diseases. Soon after its introduction, it was demonstrated that iPSCs produced

using viral or episomal vectors could be immunogenic (Zhao et al., 2011). Although immature iPSCs would never be used for transplantation in clinical settings, differentiated cells derived from iPSCs could also be immunogenic (Apostolou & Hochedlinger, 2011). It is clear that significant challenges remain despite the accumulating hopeful data regarding the therapeutic use of stem cells. The documentation of HLA (or MHC) expression by ESCs has obscured the fact that any transplanted stem cell-derived tissue, unless genetically identical to the recipient, has the potential to induce allograft rejection through "indirect" recognition (Taylor et al., 2011). Furthermore, the absence of HLA molecule expression does not guarantee immune tolerance, as natural killer (NK) cells can still be activated (Bryceson & Long, 2008). As a recent improvement, integrating a gene for a self-destruction mechanism into cells offers an extra safety measure in transplantation. This allows for the selective elimination of transplanted cells if they develop into tumors. Examples of such systems include triggers for cell death (apoptosis), the production of enzymes that convert harmless substances into toxic ones, and the expression of surface receptors that can be targeted with specific antibodies (Jones et al., 2014).

Despite the significant hurdles that remain in developing iPSC-based cell therapy, the technology offers an immense potential for treating and mitigating human diseases. Establishing clinical-grade iPSC banks with a diverse repertoire of HLA types could prove invaluable. Nakatsuji (2010) recently estimated that a collection of unique iPSC lines homozygous for the three HLA loci (A, B, and DR) could provide full HLA matching for 80–90% of the Japanese population (Nakatsuji, 2010). Additionally, Tamaoki et al. (2010) explored using DPSCs to create iPSCs banks with a sufficient HLA-type repertoire. The practical isolation and handling of dental pulp cells could facilitate expanding these banks across multiple institutions and establishing numerous iPSC lines homozygous for the three HLA loci (Tamaoki et al., 2010). While establishing stem cell banks containing pluripotent cells that are closely matched or compatible for HLA, along with inducing antigen-specific immunological tolerance instead of lifelong immunosuppressive therapy, offers hope for avoiding immunological rejection, it is crucial to consider cost management and potential cost reduction strategies when transitioning to clinical applications (Kim et al., 2022).

Cerneckis et al. (2024) thoroughly reviewed the field and concluded that as technology advances alongside iPSC research, new opportunities are emerging to better understand the molecular mechanisms of iPSC induction, refine the differentiation of iPSCs into somatic cells, develop advanced drug screening platforms, and create effective cell therapies. They anticipate that continued improvements in areas such as microscopy, multiomics, CRISPR/Cas9-based gene and protein studies, epigenetic engineering, machine learning algorithms, and other techniques will provide further insights into the molecular processes governing somatic cell reprogramming to pluripotency and iPSC differentiation into specific cell types (Cerneckis et al., 2024).

8.5 Applications of iPSCs Through Organoids

Despite the many benefits of iPSCs, including their easy accessibility, ability to replicate a patient's unique genetics, unlimited supply, and reduced risk of immune rejection when using a patient's own cells, several factors can still influence their effectiveness in tissue engineering. Yet, the most exciting development in iPSC research is seen as the application of organoids. Organoid research holds the key to fully unlocking the potential of stem cell discovery by delving into complex interactions, a departure from the simplistic linear approach I have promoted throughout this book. The challenges posed by iPSCs, including tumorigenicity and immune rejection, have necessitated more intricate research frameworks. Today, organoids represent the zenith of this investigative pursuit.

Organoids are three-dimensional cultures designed to replicate the histology, pathology, architecture, and genetic characteristics of various organs and tissues, offering an alternative to 2D cell cultures, xenografts, and animal models. These 3D in vitro models, derived from embryonic or adult tissue stem cells, closely mimic the structure and function of original tissues, such as the liver (Schutgens & Clevers, 2020). Researchers utilize diverse scaffold-based 3D cultures, including spheroids, organoids, and sophisticated "organ-on-chip" systems, to mimic the structure and function of native tissues. These scaffolds provide a supportive environment for cells to grow, interact, and self-organize into complex structures that more closely replicate the conditions found within living organisms (Mazrouei et al., 2020). Organoids are usually created from ESCs, iPSCs, and adult stem cells. These 3D structures show promise as alternatives to traditional models because they can mirror patient responses to treatments like drugs, making them particularly valuable for studying rare diseases where clinical trials are difficult. Organoids have diverse applications, including research on infectious and hereditary diseases, toxicity testing, and the potential for personalized medicine tailored to individual patients (Artegiani & Clevers, 2018; Bartfeld & Clevers, 2017; Okumura et al., 2024).

Given the right differentiation conditions, iPSCs possess the inherent ability to self-assemble into organoids. The field of organoid research has expanded significantly in recent years, with the development of numerous protocols for creating organoids that model key human organs. The kidney, brain (including specialized cortical, midbrain, hippocampal, cerebellar, retinal, etc.), fundic, and gastric organoids can develop impressive complexity. As organoid differentiation protocols become more sophisticated, they allow the creation of organoids that closely resemble specific regions of organs. A different approach to self-organizing organoids is the organ-on-a-chip (OoC). This platform involves the assembly of relevant cell types into a microfluidic device to recreate specific elements of tissue structure and function. OoC systems can be used to model tissue interfaces where compartment separation is crucial, such as the blood-brain barrier or the airway epithelium. Moreover, iPSC-derived cells and organoids can be transplanted into living organisms to create animal models with human-like features. For a more comprehensive overview, please refer to Cerneckis et al. (Cerneckis et al., 2024), an exceptionally

well-written review that is up-to-date with the latest developments in this field. Despite their potential, organoids have limitations that prevent them from fully replacing animal testing. Notably, the lack of vasculature (blood vessels) in organoids can hinder their growth and maturation, resulting in differences in behavior compared to the original tissue (Park et al., 2024). Advancements in iPSC differentiation and maturation protocols will lead to the development of effective cellular therapies, while the creation of the entire organs from iPSCs may be achievable through chimeric organogenesis. Overall, iPSC technology will continue to drive fundamental research and therapeutic advancements, accelerating scientific discovery and alleviating human diseases (Cerneckis et al., 2024).

8.6 Reprogramming of DPSCs to iPSCs

Generating pluripotent cells in vitro by "rewinding" the internal clock of somatic cells to an embryonic state, and subsequently guiding their differentiation into the desired cell types, is a promising and ongoing approach in regenerative medicine (Yildirim, 2012a). This approach can utilize readily available human tissues that pose no ethical or surgical concerns, such as fat, blood, biopsy specimens, skin, plucked hair, and extracted teeth (Aasen et al., 2008; Ali et al., 2023; Shrestha et al., 2019; Sun et al., 2009; Wada et al., 2011; Wang et al., 2023; Yan et al., 2010; Ye et al., 2009; Yildirim, 2012a).

DPSCs can be effectively reprogrammed into iPSCs (Ali et al., 2023; Atari et al., 2012; Beltrão-Braga et al., 2011; Oliveira et al., 2023; Pisal et al., 2018; Yan et al., 2010; Yildirim, 2012a, b), possibly due to their mesectodermal origin and inherent expression of reprogramming factors (Hong et al., 2023; Liu et al., 2011b; Luzuriaga et al., 2019; Peng et al., 2014; Uribe-Etxebarria et al., 2017; Yildirim, 2012a). Our preliminary results clearly indicate that DPSCs, SHEDs, and PDLSCs, exhibiting high single-cell clonogenicity, responded well to the polycistronic vector EFa-STEMCCA, which includes Oct4, Klf4, Sox2, and c-Myc (Yildirim, 2012a) (Fig. 8.1). Based on morphological criteria alone (Blelloch et al., 2007; Maherali et al., 2007; Meissner et al., 2007), dental pulp and periodontal ligament-derived iPSCs resemble ESCs: small cells in colonies with clear borders separating them from the feeder layer (Fig. 8.2). The expression of pluripotency markers in DPSCs and SHEDs was detected by RT-PCR (Fig. 8.3, top). Although Sox2 was not detected before reprogramming, it was consistently expressed throughout passages in reprogrammed dental cells (Fig. 8.3, bottom). Rapidly cycling parental dental pulp and PDL cells became smaller and continued to grow as a monolayer on irradiated human foreskin fibroblast (HFF) feeder layer cells. Although immunocytochemical analysis has not confirmed whether most cells expressed the reprogramming factors, the high colony-forming efficiency suggests that the factors successfully induced the initial morphological changes associated with reprogramming (Papp & Plath, 2013; Plath & Lowry, 2011). While these cells were positive for alkaline phosphatase, an intermediate stage marker, the continuous expression of

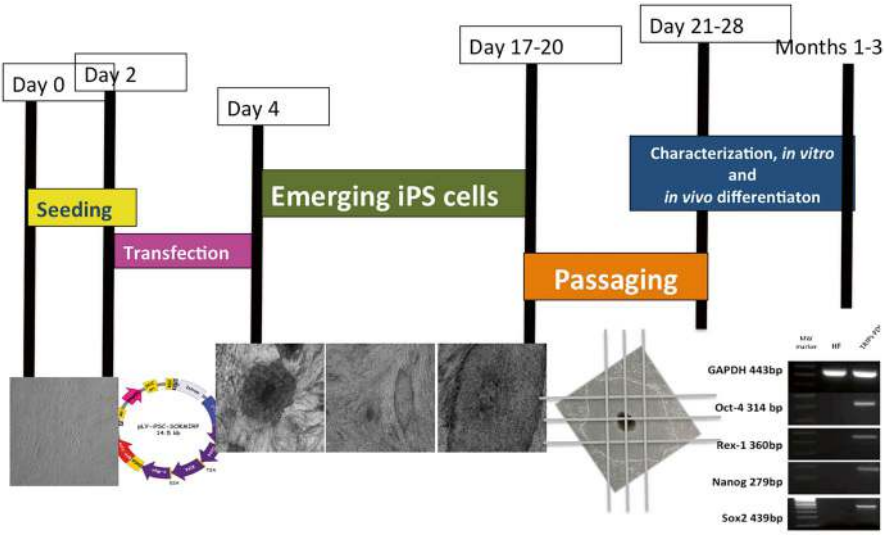

Fig. 8.1 The reprogramming process of DPSC. (Yildirim, 2012a)

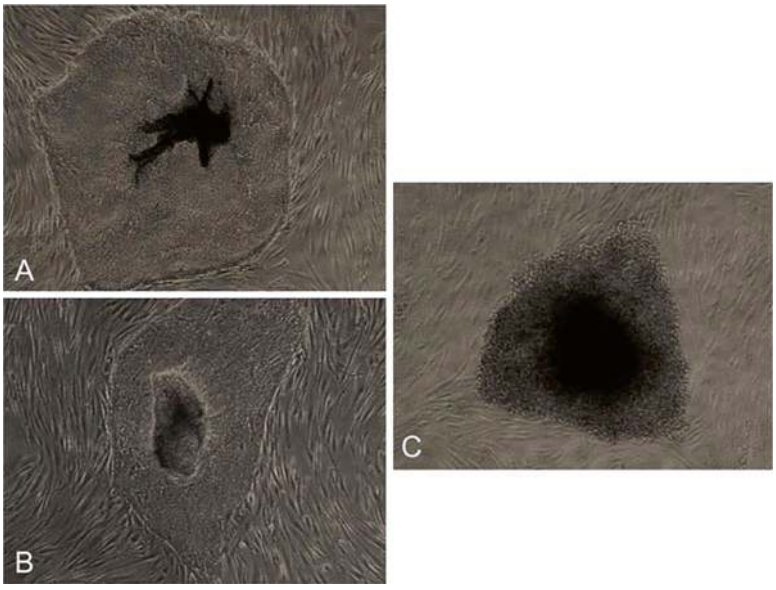

Fig. 8.2 Morphologically different iPSC colonies derived from (**A**) PDL stem cells, (**B**) SHED, and (**C**) DPSC (inverted 100×)

reprogramming factors through early passages suggests they may be good candidates to overcome barriers to pluripotency and maintain the iPS state, even independent of factor expression (Brambrink et al., 2008; Papp & Plath, 2013).

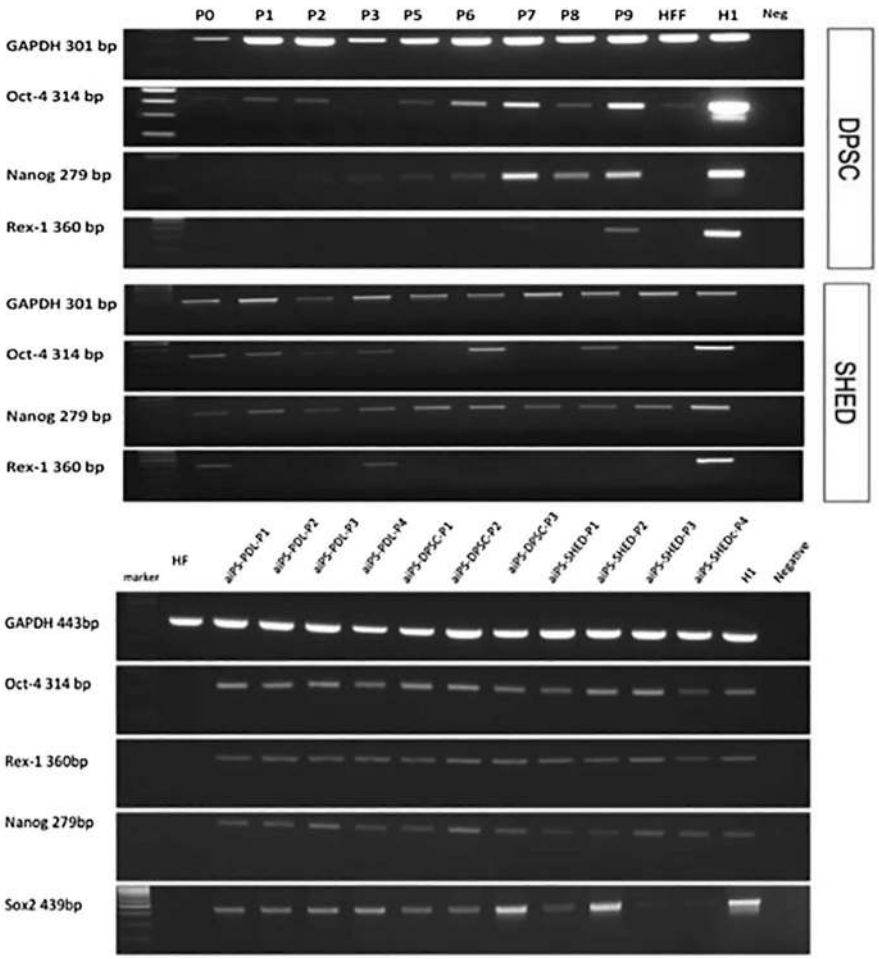

Fig. 8.3 Expressions of pluripotency markers by DPSC and SHED (on *top*) and derived iPSC (on the *bottom*) (*HFF* human fetal fibroblasts, *H1* human embryonic stem cell line) (Yildirim, 2012a)

It is interesting to note that while SHED- and PDL-derived iPSC colonies have very similar morphologies, iPSCs obtained from DPSCs display compact, dome-shaped morphologies, similar to mouse ESCs. DPSC-derived colonies have a distinct size and shape (Fig. 8.2). Since there is limited information about the biological differences between permanent and deciduous dental pulp tissues, it is difficult to explain why colonies derived from DPSCs and SHEDs are so morphologically distinct. It has been proposed that the MSC properties of PDL and DPSC are nearly identical (Huang et al. 2009), a finding consistent with the morphological similarities of SHED- and PDL-derived iPSC colonies observed in this study. The question of whether these morphological discrepancies reflect the epigenetic status of different, yet closely related, cellular origins warrants further investigation.

In the reprogramming process, genome-wide alteration of epigenetic marks and mesenchymal-epithelial transition (MET) are necessary for acquiring pluripotency, along with the regulation of checkpoints (Papp & Plath, 2013). Genetic factors, signaling pathways, and p53 pathways are potential contributors to reprogramming dynamics. It has been suggested that some, if not all, reprogramming factors might be dispensable when somatic cells express high levels of these factors (Aoi et al., 2008; Huangfu et al., 2008; Li et al., 2009b; Mao et al., 2014; Meyer et al., 2015; Tsai et al., 2010; Velychko et al., 2019). Although we did not detect Sox2 expression in our parental cells, other studies have reported the presence of key reprogramming factors, including Sox2, in DPSCs. While Liu et al. (Liu et al., 2011b) detected Oct4, Sox2, and c-Myc expression in DPSCs, Kerkis et al. (Kerkis et al., 2006) identified Oct4 and Nanog expression in these cells, consistent with our findings.

Wnt, TGF-b/activin/nodal, and BMP signaling play crucial roles in induced reprogramming (Saha et al., 2008). A downstream effector of the Wnt pathway, an inhibitor of glycogen synthase kinase-3 (GSK3), has been shown to maintain pluripotency in human and mouse ESCs (Sato et al., 2004). Additionally, Oct4 interacts with the Wnt/β-catenin signaling pathway, and these interactions are involved in maintaining stem cell integrity and regulating cell fate decisions (Abu-Remaileh et al., 2010; Qin et al., 2024). Canonical Wnt signals are transduced through the frizzled receptor and LRP5/LRP6 coreceptor, leading to the downregulation of GSK3. Our RT-PCR screening data on dental pulp tissue gene expression demonstrated that both permanent and deciduous dental pulp tissues express LRP5 and Jagged1 (see Fig. 6.9). Notably, Jagged1 has been reported as a β-catenin target gene essential for ectopic hair follicle formation in the adult epidermis (Estrach et al., 2006; Kaimari et al., 2023).

As one of the most studied pathways, TGF-b/activin/nodal plays important roles in the maintenance of the pluripotent state, and tooth development includes almost all members in this pathway (Thesleff et al., 1995). Dental pulp expresses abundantly TGF-b family members (Gu et al., 1996; Lin et al., 2017; Liu et al., 2022; Sloan et al., 1999; Yildirim et al., 2008). p53 acts as a barrier to somatic cell reprogramming (Gong et al., 2016; Kawamura et al., 2009; Krizhanovsky & Lowe, 2009; Marión et al., 2009; Utikal et al., 2009). It has been suggested that instead of completely silencing the p53 pathway, selecting target cells with naturally lower levels of p53 activity and/or higher proliferative capacity might lead to more successful reprogramming (Menendez et al., 2010; Tapia & Schöler, 2010). In the dental pulp, p53 could not be detected immunohistochemically (Piattelli et al., 2000). Dental pulp cells with high proliferative capacity and low p53 expression are likely to contribute more effectively to reprogramming events under these conditions. This can be further enhanced by inhibiting TGF-β and BMP signaling (Chng et al., 2011; James et al., 2005; Varzideh et al., 2023; Xu et al., 2008) and supplementing the culture conditions with Wnt signaling activators, such as Wnt3a (Bredenkamp et al., 2019; Cevallos et al., 2018; Marson et al., 2008; Sonavane & Willert, 2023).

8.6.1 *Is Reprogramming Necessary for Dental Regenerative Therapies?*

It has been questioned since the emergence of iPSC in cell biology, whether it is necessary to reprogram cells back to the pluripotent stem cell state for regenerative therapies or not. Pluripotency may well not be a prerequisite for the generation of certain differentiated cell types. This groundbreaking discovery of reverting the cellular clock back into the original position may help dig deeper into cellular differentiation mechanisms. Moreover, modeling many human diseases might be possible by the discovery of cell-type-specific lineage reporters, lineage-tracking tools, and tools to disrupt, repair, or overexpress genes (Saha & Jaenisch, 2009). If iPSC technology can provide a step-back system reversing the development, during which there is a gradual loss of differentiative potency proceeding from totipotency to pluripotency and multipotency in committed cell lineages toward terminal differentiation (Hemberger et al., 2009), we would meet with a ground-state model that can serve as an observation tower for differentiation and lineage commitment. Given that the dental pulp is composed of a highly diverse cell population—including connective tissue cells, specialized odontoblasts, immune cells, and a rich neuronal and vascular network—inducing these ontogenically closed cells back to a common progenitor state (possibly mesectodermal cells or neural crest cells) could offer a valuable opportunity to trace their pathways through cellular specialization. Another potential way to utilize dental pulp iPSCs is by mimicking epithelial-mesenchymal interactions. The dental pulp contains DPSC, which can differentiate to epithelial cells (Karaöz et al., 2011; Marchionni et al., 2009; Nam & Lee, 2009; Sakai et al., 2010; Zhao et al., 2012). After dissociation of epithelial and mesenchymal fractions, these cells can be reprogrammed into dental epithelial iPSC and dental mesenchymal iPSC. Since during the early passages of iPSC their epigenetic memories are retained (Kim et al., 2010a, b, 2020, 2024), these two pluripotent populations can be co-cultured with a biomimetic scaffold between them in order to obtain developmental epithelial-mesenchymal interactive events (Fig. 8.4).

Numerous research studies have demonstrated the potential of iPSCs to serve as a valuable source of dental stem cells. This is achieved by successfully transforming iPSCs into different types of dental cells. Not only have dental epithelial-like stem cells derived from human iPSCs been established (Arakaki et al., 2012; Kim et al., 2020), but neural crest MSCs derived from hiPSCs have also been reported to possess the ability to differentiate into dental cells, particularly when combined with dental epithelial cells (Kim et al., 2024). Furthermore, hiPSC-derived dental stem cells have been identified as a promising new source of cells for regenerating dental tissues using the patient's own cells (Gao et al., 2024). Currently there are efforts being made to develop tissue-engineered tooth-like structures using mouse iPSC (Wen et al., 2012). It has been reported that the unlimited iPSC-induced neural crest-like cells (iNCLCs) are a promising cell source for tooth development and dental tissue/tooth organ regeneration studies (Otsu et al., 2012). When transplanted in vivo with a tooth scaffold, these iNCLCs formed a dentin-pulp complex.

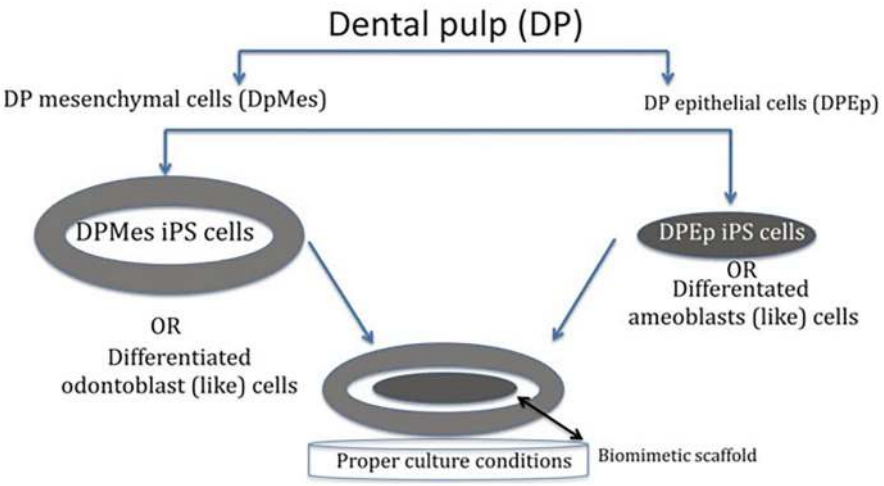

Fig. 8.4. A working hypothesis for epithelial-mesenchymal interactions to mimic dental development

Odontoblast-like cells derived from the iNCLCs formed a single layer on the surface of the pulp-like tissue beneath the newly formed dentin, mirroring the structure of the normal dentin-pulp tissue in a mouse molar (Zhang et al., 2020b). Similarly, when iPSCs-based neural crest-like cells were briefly treated with FGF4 and FGF9 and then grafted into the backs of mice, they replicated the characteristics of the dental pulp tissue. This included the presence of odontoblast-like cells, a dentin-like layer, and significant new blood vessel formation induced by the angiogenic factors SAPH and SLan (Kobayashi et al., 2022).

While there is still much to learn about the nature, potential, and activity of dental stem/progenitor cells, their potential for use in dental tissue regeneration is immense and could revolutionize the treatment of dental diseases. Consequently, these cells have been widely used in animal tissue engineering studies to explore their potential for preclinical applications (Wang et al., 2022). However, despite a significant progress in stem cell research, their effectiveness and applicability in clinical trials remain uncertain.

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Chapter 9
Need for Tangible Methods for Clinical Translation

Arnold Caplan, the pioneering figure behind the discovery of mesenchymal stem cells (MSCs), has recently passed away. His contributions to stem cell science were monumental, being instrumental in the clinical translation of MSCs. However, Caplan later acknowledged the limitations of the prevailing dogma of the late 1980s that "what we saw in cell culture is what happens *in vivo*," recognizing that this oversimplified view had influenced his research and, consequently, clinical applications (Caplan, 2021). He proposed the term "medicinal signaling cell" as an alternative, admitting that MSCs might not differentiate into mesenchymal cells in vivo.

Unfortunately, the linear thinking embedded in many clinical applications has persisted. Numerous scientifically questionable "treatments" under the banner of stem cell therapy are now customary worldwide. These practices align with the criticisms we have previously articulated in the first chapter. While clinicians and researchers who design studies based on similar assumptions bear some responsibility, so too do the patients eager for quick and easy solutions. This psychology of seeking immediate relief can be attributed to the concept of "post-truth," where objective facts are less influential in shaping public opinion than appeals to emotion and personal belief.

The content in this chapter might seem convoluted and difficult to grasp. My aim is to brainstorm why we still have not reached effective treatments in stem cell and cancer research, despite nearly a century or more of progress. Even with the relationships we have tried to establish through Newtonian and neo-Darwinian reductionist perspectives, and mostly through linear thinking, the point we have reached is actually remarkable. However, I believe that multidisciplinary research encompassing other scientific fields, especially mathematics and physics, will enable us to deal with the realities that are right in front of us.

S. Yildirim, *Dental Pulp Derived Mesenchymal Stromal Cells*,
https://doi.org/10.1007/978-1-0716-4244-3_9

9.1 Abandoning the Stem Cell Term

In Chap. 1, we discussed the lack of consensus regarding nomenclature. According to Merriam-Webster, a name is "a word or phrase that constitutes the distinctive designation of a person or thing." Therefore, when we refer to nomenclature, we are also referring to the distinctive designation of the subject in question.

In the past decade, research extensively explored the potential of MSCs to regenerate various tissues beyond their expected skeletal origins. This idea extended to tissues derived from other embryonic layers, leading to the concept of MSCs as versatile "plastic" cells similar to embryonic stem cells. In vitro studies claimed to efficiently transform MSCs into different cell types using chemical signals, fueling preclinical and clinical trials. However, these studies often lacked robust methods to confirm true cellular transformation in artificial lab conditions and primarily relied on observational changes in cell behavior. This approach contradicted the established understanding that adult stem cells are restricted in their development potential. Applying methods suitable for highly flexible embryonic stem cells to more specialized adult cells was inappropriate. Inducing specific changes in adult stem cells is more accurately described as reprogramming rather than direct differentiation (Bianco et al., 2013).

Rodeo (Rodeo, 2021a) clearly articulated the problems in his editorial piece that:

> "The term "stem cell" is perhaps one of the most over-used terms in the orthopaedic literature. Caplan introduced the term "mesenchymal stem cells" to the orthopaedic vernacular in a classic 1991 article. The term conjures up images of cells that can support the formation of a variety of new tissues after transplantation. However, true tissue formation in vivo using culture-expanded cells has proven to be elusive. The term "stem cells" has been widely misused in aggressive advertising and marketing, implying promise that has largely not been demonstrated in preclinical and clinical studies. At the same time, positive effects of cell transplantation are certainly known to occur, even without long-term engraftment or survival of transplanted cells."

Rodeo (Rodeo, 2021b) later has provided a comprehensive overview of the current challenges associated with MSCs in response to Caplan's apology (Caplan, 2021):

1. Transplanted cells do not differentiate.
2. Transplanted cells do not engraft.
3. Transplanted cells function via a paracrine effect ("medicinal signaling cells").
4. There are essentially zero "stem cells" in adult tissues.

According to Rodeo (2021b), most organs contain a small population of specialized cells with a limited potential to develop into specific cell types. These cells aid in local tissue repair following injury. He proposes the term "connective tissue progenitors" for cells originating from adult tissues such as bone marrow, fat, and blood. These cells do not possess the full characteristics of true stem cells, including the ability to continuously renew themselves, produce identical daughter cells, and develop into multiple cell types. I agree with this and previously discussed it in the context of DPSCs. Rodeo suggested that we should even avoid using the term "stem cell" altogether (Rodeo, 2021b).

At this juncture, I strongly recommend reading Sipp et al.'s articles, "Clear Up This Stem Cell Mess" (Sipp et al., 2018), MacArthur's "Stem Cell Biology Needs a Theory" (MacArthur, 2023), and Bianco et al.'s "The meaning, the sense and the significance: translating the science of mesenchymal stem cells into medicine" (Bianco et al., 2013), which were also extensively referenced in Chap. 1. These, and similar articles, discuss the prevalence of unscientific treatments labeled as "stem cell therapies," as well as the reasons why we have not yet achieved effective and truly scientific treatments in this field when the aforementioned criteria are not considered. Foremost among these realities is our mindset regarding health and disease. With our deep-rooted belief in disease, we might believe that the treatment should be simple, as if the occurrence of these diseases were simple. This leads us down a path I call "post-truth medicine." Here and now, let us examine the concepts of health and disease, mostly with the quotations of great minds.

9.2 Health vs. Disease

Transplantation of corrected or differentiated stem cells into a diseased human tissue is likely the most challenging issue for the prognosis of stem cell technology. The ultimate and most desired goal in regenerative medicine is to replace the dead or non-functioning cells causing disease symptoms with healthy, laboratory-produced cells (Wilmut et al., 2011). Although we can now obtain large quantities of cells and tissues from specific patient populations, it remains unclear whether cells derived from complicated technologies can effectively reconstitute tissues at diseased sites (Saha & Jeanish, 2009).

Very contemporary insights into evolutionary theory and complexity are incorporated in Goodwin's aforementioned book (Goodwin, 1996). He defines organisms as:

> "Organisms are endowed with powerful particulars that give them the capacity to regenerate and reproduce their own natures under particular conditions, whereas inanimate systems cannot. This is an emergent property of life that is not explained by the properties of the molecules out of which organisms are made, for molecules do not have the capacity to make a whole from a part. DNA and RNA can make copies of themselves under particular conditions, but this is a self-copying process, not one in which a more complex whole is generated from a part. This is a principal reason organisms cannot be deduced to their genes or molecules. The particular type of organization that exists in the dynamic interplay of the molecular parts of an organism, which I have called a morphogenetic or a developmental field, is always engaged in making and remaking itself in life cycles and exploring its potential for generating new wholes.… We have now recovered organisms as the **irreducible** entities that are engaged in the process of generating forms and transforming them by means of their particular qualities of action and agency, or their causal powers. This includes hereditary particulars that give organisms a type of memory, and the intimate relations of dependence and influence between organisms and their environments. The life cycle includes genes, environmental influences, and the generative field in a single process that closes on itself and perpetuates its nature generation after generation. Species of organisms are therefore natural kinds, not the historical individuals of Darwinism. The members of a species express a particular nature."

The fact that the organisms/tissues/organs or even a cell are irreducible entities and moreover there is breathtaking orchestration between all and every part of the organism raises deeper questions about whether the human body can truly be treated like a machine, with broken parts simply replaced by spare ones.

Goodwin's statements parallel to German philosopher Immanuel Kant on the distinction between mechanisms and organisms are legendary: "Kant described a mechanism as a functional unity in which the parts exist for one another in the performance of a particular function. An organism, on the other hand, is a functional and a structural unity in which the parts exist for and by means of one another in the expression of a particular nature. This means that the parts of an organism –leaves, roots, flowers, limbs, eyes, heart, brain– are not made independently and then assembled, as in a machine, but arise as a result of interactions within the developing organism. So, organisms are not molecular machines. They are functional and structural unities resulting from a self-organizing, self-generating dynamic. The emergent qualities that are expressed in biological form are directly linked to the nature of organisms as integrated wholes, which can be studied experimentally and stimulated by complex nonlinear models." (Goodwin, 1996).

Furthermore, Goodwin explains how our realization of a human being influences the way of treating the illness:

> "If humans are to be understood essentially in terms of genes and their products, then illness is to be corrected by manipulating them. The result is drug-based medicine and genetic counseling or engineering. These can be extremely effective in certain circumstances, but medical care based on this approach focuses on illness rather than on health. Organisms and persons are not the effect of molecular and neuronal causes, of genes and traits, but instances of the unfolding of the total relational field. They are formed from relationships, which in their activities they create anew" (Goodwin, 1996).

Such novel insights need to overcome strong resistance from cell biologists who are very loyal to conventional theories of life. Fortunately, these new ideas are being presented using simple mathematics and physics as much as possible.

9.3 Changing Concepts in Cell Biology

Claire Colebrook (Colebrook, 2002) argues that we are in a post-linguistic era, necessitating the development of theories and approaches not reliant on language. However, we have been firmly committed to using language exclusively to describe the immensely complex and interactive events in biology. If we believe that mathematics is the language of nature, then we should be able to represent and comprehend everything around us through numbers. When these numbers, derived from any system, are translated into graphs, patterns emerge. Consequently, patterns are ubiquitous in nature (Aronofsky, 1998). The way complex systems and patterns arise from a multitude of relatively simple interactions is known as emergence. Thus, emergence is central to theories of integrative levels and complex systems (Corning, 2002). However, in the twenty-first century of science, I believe we are

like colorblind individuals in biology, reminiscent of the didactic story by Leon Lederman and Dick Teresi in their book *The God Particle* (Lederman & Teresi, 1993). In this story, intelligent beings from the imaginary planet Twilo, who cannot perceive sharp black and white contrasts, come to earth on a goodwill mission. To test their understanding of our culture, their hosts take them to a World Cup soccer match, the most popular cultural event on the planet. Unable to see black and white, the Twilo watch the game with polite but bewildered expressions. Lederman continues:

> "As far as the Twiloans are concerned, a bunch of short-pantsed people are running up and down the field kicking their legs pointlessly in the air, banging into each other, and falling down. At times an official blow a whistle, a player runs to the sideline, stands there, and extends both his arms over his head while the other players watch him. Once in a great while the goalie inexplicably falls to the ground, a great cheer goes up, and one point is awarded to the opposite team.
>
> The Twiloans spend about 15 min being totally mystified. Then, to pass the time, they attempt to understand the game. Some use classification techniques. They deduce, partially because of the clothing, that there are two teams in conflict with one another. They chart the movements of the various players, discovering that each player appears to remain more or less within a certain geographical territory on the field. They discover that different players display different physical motions. The Twiloans, as humans would do, clarify their search for meaning in World Cup soccer by giving names to the different positions played by each footballer. The positions are categorized, compared, and contrasted. The qualities and limitations of each position are listed on a giant chart. A major break comes when the Twiloans discover that symmetry is at work. For each position on Team A, there is a counterpart position on Team B.
>
> With 2 min remaining in the game, the Twiloans have composed dozens of charts, hundreds of tables and formulas, and scores of complicated rules about soccer matches. And though the rules might all be, in a limited way, correct, none would really capture the essence of the game. Then one young pipsqueak of a Twiloan, silent until now, speaks his mind. "Let's postulate," he ventures nervously, "the existence of an invisible ball."
>
> "Say what?" reply the elder Twiloans.
>
> While his elders were monitoring what appeared to be the core of the game, the comings and goings of the various players, and the demarcations of the field, the pipsqueak was keeping his eyes peeled for rare events. And he found one. Immediately before the referee announced a score, and a split second before the crowd cheered wildly, the young Twiloan noticed the momentary appearance of a bulge in the back of the goal net. Soccer is a low-scoring game, so there were few bulges to observe, and each was very short-lived. Even so, there were enough events for the pipsqueak to note that the shape of each bulge was hemispherical. Hence his wild conclusion that the game of soccer is dependent upon the existence of an invisible ball (invisible, at least, to the Twiloans).
>
> The rest of the contingents from Twilo listen to this theory, and, weak as the empirical evidence is, after much arguing, they conclude that the youngster has a point. An elder statesman in the group—a physicist, it turns out—observes that a few rare events are sometimes

> more illuminating than a thousand mundane events. But the real clincher is the simple fact that there must be a ball. Posit the existence of a ball, which for some reason the Twiloans cannot see, and suddenly everything works. The game makes sense. Not only that, but all the theories, charts, and diagrams compiled over the past afternoon remain valid. The ball simply gives meaning to the rules.
>
> This is an extended metaphor for many puzzles in physics, and it is especially relevant to particle physics. We can't understand the rules (the laws of nature) without knowing the objects (the ball) and, without a belief in a logical set of laws, we would never deduce the existence of all the particles" (Lederman & Teresi, 1993).

We may possess such an "alien" perspective within the unfathomably complex system of cell biology. To gain a "native" understanding, we need to think and see differently, to perceive all the colors and shapes. There might be several ways to achieve this, but none could be as vibrant and meaningful as what Mandelbrot brought to our sight. Mandelbrot recognized the endless repetition of self-similarity in many biological forms, such as a tree, where each pattern of branching is very similar. His seminal book, *The Fractal Geometry of Nature*, was published in 1982 (Mendelbroth, 1982). This maverick mathematician passed away at 85, leaving behind fractal geometry, which has been applied to physics, biology, finance, and many other fields. His own description of himself summarizes his contribution to science: "I decided to go into fields where mathematicians would never go because the problems were badly stated. I have played a strange role that none of my students dare to take."

Looking at the picture in Fig. 9.1, although it is black and white, I can't help but think how much mathematical sense and foresight we need in biology.

Would it be possible to engineer cells layer by layer? Could we explain all the events at every level of that construction using fundamental mathematics and physics? Goodwin, in his book *How Leopard Changed Its Spots*, defines the unification of biology, physics, and mathematics (Goodwin, 1996):

> "The result is a unification of biology, physics, and mathematics that is accelerated by studies in the sciences of complexity and the realization that similar types of dynamic behavior arise from complex systems, irrespective of their material composition and dependent primarily on their relational order –the way the parts interact or are organized. Biology thus becomes more physical and mathematical, putting the insights of genetic, developmental, and evolutionary studies into more precise dynamical terms; at the same time, physics becomes more biological, more evolutionary, with descriptions of the emergence of the four fundamental forces during the earliest stages of the cosmic Big Bang, the growth sequences of stars, and the formation of the elements during stellar evolution. Instead of physics and biology remaining opposites, the former seen as the science of rational order deduced from fixed laws of nature and the latter described (since Darwin) as a historical science, physics is becoming more evolutionary and generative while biology is becoming more exact and rational."

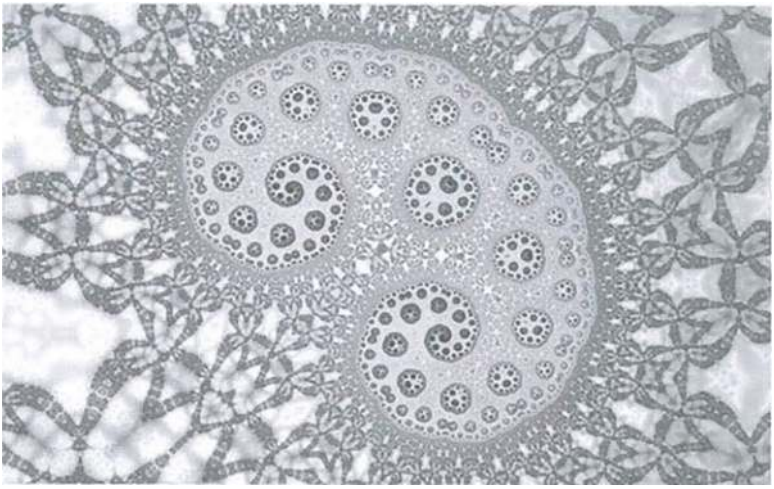

Fig. 9.1 The picture is a result of the fractal formula of z = (z*c + 1) + 1/(z*c + 1) (Softoblog, 2011)

9.4 A New Theory Is Yet to Come

While a unified theory of MSC function has not yet been established, current approaches do not fully explain the clinical utility of these cells and leave several questions unanswered. Emerging evidence from new experiments, observations, and research may challenge or contradict the existing understandings. In this context, developing a comprehensive theory that addresses these limitations and incorporates new evidence is crucial for advancing the field.

MacArthur (MacArthur, 2023) is one of the pioneers who articulated a theory for stem cells. According to MacArthur, "In scientific disciplines like mathematics and physics, theories are frequently articulated through mathematical equations, forming models that can be simulated computationally. However, a theory does not necessarily require mathematical formulation. It can simply be a collection of statements or principles that elucidate a set of data, as exemplified by Darwin's prose-based theory of evolution through natural selection. Considering this, we can ponder: what constitutes a good theory within the realm of stem cell biology?"

MacArthur suggests five principles for such a theory:

1. Parsimonious yet complete: The theory should be concise and include only the essential elements necessary to explain the phenomena, avoiding unnecessary complexity.
2. Mechanistically insightful: The theory should not only describe observations but also provide a deep understanding of the underlying mechanisms and causal relationships.
3. Experimentally generative: The theory should be testable and capable of guiding the design of new experiments that can further validate or refine it.

4. Unifying and integrative: The theory should connect seemingly disparate observations and data into a coherent framework, revealing underlying patterns and relationships.
5. Generalizable and broadly applicable: The theory should transcend specific cases and provide a general framework that can be applied to a wider range of phenomena (MacArthur, 2023).

Sometimes, it helps consider a different perspective. Looking at an existing theory from a new angle or trying a different approach can lead to a fresh theory. This new theory might explain the same phenomenon in a new way or offer different predictions. In essence, as Ralph Waldo Emerson wisely stated, "Do not follow where the path may lead, go instead where there is no path and make a trail."

Huang et al. (2005) presented the concept of cell types as high-dimensional attractors. The transition from linear genetic pathways to gene regulatory networks reveals a dynamic trajectory within a complex state space, where each point represents a network state and corresponding cell state (Huang, 2009). A common idea in the field is that different cell functions correspond to "attractors," which are stable or balanced states within complex systems. These systems are controlled by intricate networks involving interactions between genes, their products, and environmental factors (Huang et al., 2005).

Huang (Huang, 2011) expanded on the idea that cell types can be seen as attractors within the epigenetic landscape, a concept that represents the relationship between the genome and complex phenotypes. This framework uses a simplified model (Fig. 9.2) where the collective behavior of regulatory genes creates a network of defined interactions, visualized as a landscape. These interactions, encoded within the genome through molecular interactions like transcription factor binding,

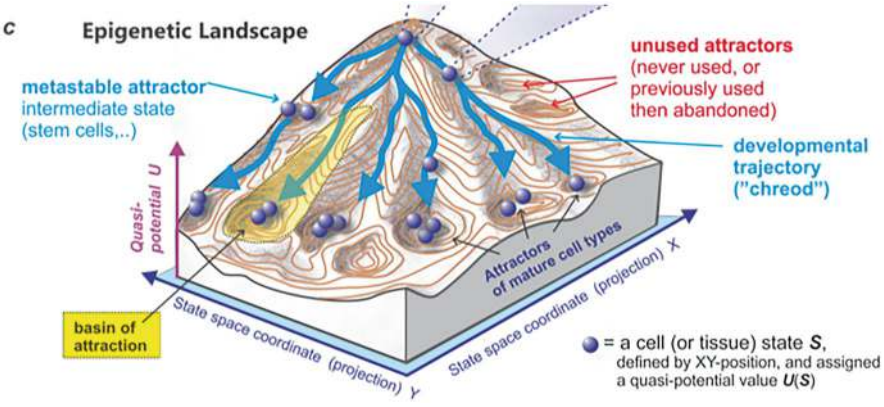

Fig. 9.2 The collective behavior of all the regulatory genes forms a network of defined regulatory interactions, and those can be displayed as a landscape. The purple balls represent cells, and the blue arrows denote the "streamlined" developmental paths from the apex attractors (embryonic pluripotent cells) to the attractors of the terminally differentiated states at the bottom of the landscape (Yildirim & Huang, 2018)

make up the "wiring diagram" of the gene regulatory network (GRN). Although the GRN is encoded in the genome, this does not imply a fixed phenotype. Understanding this distinction is crucial for grasping developmental plasticity (Yildirim & Huang, 2018).

MacArthur (MacArthur, 2023) wraps up the discussion by suggesting a straightforward solution: "To truly understand stem cell fate and function, we need to leverage tools from mathematics, physics, and computational sciences. This interdisciplinary approach will help us uncover the universal principles governing stem cells and gain a better understanding of the complex cellular and molecular mechanisms that regulate them."

9.5 Post-Truth Medicine

Post-truth refers to a situation in which emotional or personal beliefs have more influence on public opinion than objective facts. In a post-truth context, subjective feelings and appeals to emotion often overshadow empirical evidence and factual accuracy in shaping public discourse and decision-making (Harsin, 2018). To internalize this concept, we can think in terms of the obsolescence of truth. The term "post-truth" was first used by Serbian-American playwright Steve Tesich in 1992. In his 1992 article "Government of Lies," he states that a significant portion of the American public accepted the political propaganda of the Bush administration as truth without question. Tesich criticizes how people no longer seek the truth but instead accept the raw information presented to them without scrutiny (Tesich, 1992). In 2016, the Oxford Dictionaries selected "post-truth" as the "Word of the Year" and defined it as "a term relating to or denoting circumstances in which objective facts are less influential in shaping public opinion than appeals to emotion and personal belief" (Oxford Dictionary, 2016).

Identifying stem cells in the hematopoietic system was considered a harbinger of a medical revolution, particularly when it was realized that these cells were also present in the non-hematopoietic stromal cells of the bone marrow. However, as of the summer of 2024, nearly 100 years since the initial discovery of stem cells, these so-called "magic" cells have failed to fulfill the potential expected of them. The culprit for this situation was not the cells themselves, but rather the scientists who attributed meanings to these cells that they did not possess. In this era where we are aware that the development trajectory of medicine focuses on diseases rather than their causes, the emphasis shifted to how diseases could be treated rather than their origins. The human body was first divided into organs and then into the building blocks of those organs. The notion emerged that if the secrets of the smallest building blocks could be unraveled, a diseased organ could be replaced with a new one, spreading throughout medicine like an illness. The view that the human body is the most magnificent system on earth gave way to the belief that its parts could be reflected in the whole. The magnificent orchestration was disregarded. Thus, stem cells became the cornerstone of a cycle where spare parts of the human body could

be manufactured in the laboratory. At this point, far fewer diseases than expected can be treated with such therapies.

In a world where lies are presented as truth, what is the meaning of truth? Around the early 2000s, concerns began to emerge regarding the use of MSCs and their applications. Pioneering voices emerged, taking on the responsibility of raising awareness among scientists and doctors as we discussed so far. Their critiques challenged the notion that simply injecting MSCs intravenously would be a miraculous solution. They argued that these cells wouldn't necessarily differentiate as intended or recognize and repair damage once within the body's complex environment. However, their efforts have not yet translated into a widespread change. As of today, clinicaltrials.gov lists thousands of research studies registered under the umbrella of stem cell therapy, highlighting the continued focus on this area despite the critiques.

Heinrich (Heinrich, 2020) recently argues that the credibility of science and its public image are undermined by scientific misconduct and the proliferation of predatory journals. These issues are exacerbated by the broader societal trend of diminishing trust in factual information. These challenges have profound implications for the field of medicine and the public's understanding of scientific matters. Furthermore, in our current era where truth is increasingly questioned, the dissemination of false research through predatory journals and dishonest authors poses a severe risk to patient health and life. The rapid spread of misinformation on social media exacerbates this issue as the public's trust in factual information declines. While the scientific community has implemented safeguards to mitigate the publication of false findings, it is crucial that every individual involved in the research process fulfills their responsibilities. Upholding the core values that have defined scientific inquiry is essential in this endeavor (Heinrich, 2020).

In closing, given my fascination with complexity science, I offer a new perspective by theorizing that dental pulp operates as a complex adaptive system.

9.6 The Dental Pulp as a Complex Adaptive System

The central dogma of molecular biology states that the molecular basis of genetic inheritance is established by a linear relationship between genetic information encoded in DNA and translated protein (DNA➔mRNA➔protein) (Huang, 2012; Li & Xie, 2011). However, with the aid of advanced single-cell fluorescence microscopy, transcriptomes and proteomes have been quantified with single-molecule sensitivity showing that the mRNA copy number does not correlate with the protein copy number for the same gene (Taniguchi et al., 2010; Yu et al., 2006). There is accumulating evidence showing that genotype might not be translated in an obvious manner into a corresponding phenotype and genes are not even the sole basis of inheritance. The intricate relationship between genotype and phenotype has become undeniably clear (Huang, 2012).

Since many phenomena in life are neither linear nor able to be reduced to their simplistic units (or both), the inability of linear analysis to explain nonlinear

systems has been accepted at the beginning of the 1900s. A nonlinear system is more than the sum of its parts, small inputs can have large system effects (or vice versa), and there is sensitivity to initial conditions, which makes prediction almost impossible (Waldrop, 1992).

Nonlinear systems have been challenging to work with because they often exceed human computational capabilities. When nonlinear relationships are identified, the relevant data might be unavailable or the complexity of the relationships makes them difficult to measure or calculate. Scholars tend to favor simpler, linear models, resulting in a field that is disconnected from real-world complexities and lacks predictive power. This preference for linear models stems from the difficulty of modeling the nonlinear outcomes of numerous interacting components (Aydinoglu, 2010; Bolourchi et al., 2015).

Analyzing a part of a system in isolation leads to flawed results because it neglects the interactions and feedback loops between different components. These feedback loops can significantly influence the behavior of the system, either amplifying or dampening certain effects, and play a crucial role in maintaining the system's equilibrium (Aydinoglu, 2010).

A complex adaptive system on the other hand consists of a large number of agents, each of which behaves according to some set of rules. These rules require the agents to adjust their behavior to that of other agents. In other words, agents interact with, and adapt to, each other (Stacey, 2003). These statements can be effectively applied to "dental pulp organization" since dental pulp behaves like any complex adaptive system (CAS) and demonstrates the basic features of such systems. The characteristics and principles of CAS are components/agents, variation and diversity, interaction and interdependency, feedback, unpredictability and nonlinearity, the edge of chaos, self-organization and emergence, adaptation and learning, historicity and path-dependence, and finally coevolution (Holden, 2005). Although regenerative, molecular, microbiological, tissue organization, and several other aspects of a tooth system should be analyzed individually, I will attempt to demonstrate how dental pulp conform to the basic features of CAS:

1. Large numbers of components: Dental pulp has various cells and tissues.
2. Variation and diversity: Each cell different from each other and its performance depends on the other cells.
3. Interaction/connectivity/interdependence: Homeostasis established via interaction/connectivity of the different elements of a dental pulp system. However, every single part of the tissue has interdependent cells (odontoblasts, fibroblasts, immune cells, etc.).
4. Feedback: Positive (amplifying) feedback to the dental pulp occurs during physiological functions of the tooth, while negative (dampening) feedback is sent via infected dentinal tubules.
5. Unpredictability and nonlinearity: Caries *may* develop in conjunction with poor oral health, or reactionary dentinogenesis *may* be triggered by the onset of caries.
6. The edge of chaos/far from equilibrium: Being away from the equilibrium (through poor oral health or continuous trauma) might alter the environment

within the tooth, creating a configuration that increases the likelihood of its survival (such as triggering reactionary dentinogenesis).
7. Emergence, self-organization-strange attractors: Reparative dentin emergence cannot be reduced to a single molecule. Many constituents have many diverse functions along the process.
8. Adaptation to the environment: Teeth are quite resilient to threats and adaptive to their environment. There is a dynamic between health and disease.

In conclusion, by utilizing emergent concepts of complexity, we can explore the properties and characteristics of dental pulp functions from a complex adaptive systems perspective. Identifying the desired dentin-specific marker(s) to characterize the replacement cell population responsible for forming reparative dentin remains challenging, as the traditional linear gene-protein model is giving way to a more nuanced understanding of complex biological systems.

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